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PHYSIOLOGICAL ZOÖLOGY

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THE SOURCE AND ACTIVITY OF CRAGO-DARKENING HORMONE (CDH)[1]

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Department of Zoölogy, Northwestern University, and Marine Biological Laboratory, Woods Hole, Massachusetts

THE common shrimp, *Crago*, shows striking and rapid color changes in response to changes in background. The range of color change varies from very pale gray-white through darker grays to purple-black. At a slower rate *Crago* may also mimic the tint of any background other than a blue or green one (Koller, 1927). In addition to these changes, which occur uniformly over the whole body, there may be exhibited a mottled coloration of the body as a result of the production of darker and lighter areas. The telson and uropods may show their whole gamut of color change in a normal animal quite independently of the color of the remainder of the body, being sometimes very dark while the remainder of the body is light, or pale while the remainder of the body is dark; but usually these appendages tend to follow the color of the rest of the body. Although the color changes are usually adaptive, rendering the shrimp roughly the shade of the background upon which it comes to lie, they may, in a small percentage of any given group of animals at any given instant, show little or no relation to background or to light-intensity

Koller (1925, 1927), working on *Crago*, gave us the first good evidence that color changes in crustaceans are hormonally controlled, by discovering that blood, transfused from a dark-colored animal to a light one, resulted in darkening of the latter. Blood from a light-colored animal did not do likewise, nor did it lighten a dark-colored animal. Despite these observations, the first well-established source of a color-change hormone in shrimp (Perkins, 1928; Koller, 1928), located in the eyestalks, yielded a principle the action of which lightened dark shrimp. However, Koller also stated at that time that extracts of the rostral region of *Crago* darkened the bodies of light animals and that destruction of a specific region therein resulted in a permanently lightened condition of the body. Koller believed that this region contained an endocrine gland, which he named the "Schwarz-organ." The gross

[1] This investigation was supported by a research grant from the Graduate School of Northwestern University.

color changes of the shrimp were believed to be controlled jointly by the lightening hormone arising in the eyestalks and by the darkening hormone from the rostral region. Since Koller's work there has been abundant confirmation of the lightening action on shrimp of an eyestalk hormone but a general failure to confirm the existence of a body-darkening one arising in the rostral region. Most recent failures to confirm the "Schwarz-organ" are those of Kleinholz (1938), Panouse (1941), and Carstam (1942).

Numerous observations, including Koller's initial blood-transfusion work (1927) and also his later observations and experiments (1930) on color changes of *Crago*, provided strong evidence that a body-darkening hormone must originate somewhere in the shrimp outside the eyestalks. Brown (1939) observed that eyestalkless specimens of *Crago*, whose bodies were generally light though somewhat mottled, showed a strong transitory darkening of the whole body in response to stimulation of the eye stubs with a hot cautery needle. The darkened condition usually lasted about an hour. This reaction seemed reasonably explained in terms of a body-darkening hormone that originated in some part of the body other than the eyestalks and was liberated as a result of stimulation of the stubs of the optic nerve. In 1940 Brown and Ederstrom, attempting to repeat this latter observation, quite consistently obtained a different result. They found that stimulation of the eye stubs by a hot cautery needle produced rapid and thorough darkening of the originally light telson and uropods and simultaneously a lightening of the remainder of the body. The opposite response of the bodies in the two foregoing experiments has since been shown (Brown and Wulff, 1941) to be explainable—in part at least—to differences in the strength of the stimulus. A stronger stimulus generally yielded a transitory lightening of the body; a weaker one, a darkening. Brown and Ederstrom, by nerve-transection experiments, demonstrated that a blood-borne agent was responsible for the transitory darkening (maximally dark in 5–10 minutes; light again in 30–80 minutes) of the telson and uropods. They extracted various tissues of the body and injected them in an attempt to locate the site of origin of the hormonal substance involved. This source was found to lie within the central nervous system. The circumoesophageal connectives showed the activity to a great degree; the rest of the nervous system seemed generally inactive. Further localization placed the site more specifically in a region of these connectives which included the connective ganglion and a short region of the connective just posterior to it. Extracts of this region both strongly lightened the body and darkened the telson and uropods—a response like that obtained by strong stimulation of the eye stubs in eyestalkless animals. Even the time-relations of the responses were very much the same in the two. These investigators also reported, regarding the darkening principle for the telson and uropods, that "further brief experiments gave us strong reason to suspect the commissure immediately posterior to the commissural ganglion to be most active." This last statement, together with the fact that about once in every ten experiments the extracts were without effect, led to an attempt at more specific localization of the source of this principle, the results of which are reported in the present paper.

The simultaneous darkening, or lightening, of the body proper and the darkening of the telson and uropods upon ap-

propriate stimulation of the eye stubs of eyestalkless animals described above were explained by Brown and Wulff (1941) by the hypothesis that two hormones were secreted within the central nervous system—one lightening all the body except the telson and uropods, and the other darkening the telson and uropods and, in the absence of the first hormone, darkening the rest of the body as well. They were able to obtain the body-lightening principle free from the "tail"-darkening one (alcohol extraction) but were unable to obtain the "tail"-darkening principle free from body-lightening activity. The present work includes further attempts to test this hypothesis.

EXPERIMENTS AND RESULTS

All the following experiments were conducted upon *Crago septemspinosus*, collected in the vicinity of Woods Hole, Massachusetts. The animals were kept in large dark-bottomed tanks of running sea water between the time of their capture in the field and their utilization in experiments.

First, a further study was made of the effects of injection of extracts of the circumoesophageal connectives, including the connective ganglia. Numerous extracts were made of pairs of connectives, and these extracts were tested in each case by injection into two *Crago* individuals which had been eyestalkless for at least 24 hours. These eyestalkless shrimp used as assay animals invariably possessed completely blanched telson and uropods and a somewhat darkened and mottled body, as illustrated by the specimen on the left in Plate I, *A*. The connectives for the experiments were removed as follows: The dorsal portion of the carapace was removed by inserting the blade of a scalpel horizontally beneath the rostrum and cutting posteriorly with a slicing movement. The animal was then eviscerated with blunt forceps, and the connectives were severed by fine scissors at the point of their junction with the supra-oesophageal ganglia. The connectives were then readily pulled backward and removed along with the thoracic chain of ganglia.

Extracts of the connectives (concentration: 1 pair of connectives to 0.2 ml. of sea water), when injected in a dosage of 0.03 ml. per specimen, gave quite variable results from both qualitative and quantitative standpoints. These results may be summarized thus: there was always a darkening of the telson and uropods, though sometimes this response was somewhat weaker than at others. Table 1 includes the results obtained from twenty-seven separately prepared extracts, each assayed upon two animals. The extent of darkening was described by values ranging from 0 to +4. The results have been averaged in four groups of six or seven experiments each. These

TABLE 1

RESPONSE OF TELSON AND UROPODS OF EYESTALKLESS ANIMALS TO INJECTION OF EXTRACT OF CIRCUMOESOPHAGEAL CONNECTIVES

Concentration: 1 pair of connectives per 0.2 ml. sea water; dosage: 0.03 ml. per animal. The sign + indicates a darkening; and the number, the relative degree of darkening, compared with the entirely light similar parts of control animals without eyestalks.

Time (Min.)	(1) Av. of 7	(2) Av. of 7	(3) Av. of 6	(4) Av. of 7
0	0.0	0.0	0.0	0.0
5	+2.2	+2.5	+2.3	+2.4
10	+3.0	+3.4	+3.8	+3.6
15	+3.4	+3.5	+3.6	+3.4
20	+3.5	+3.5	+3.5	+3.4
40	+2.4	+2.3	+1.6	+1.6
60	+1.8	+1.0	+0.2	+0.1

four groups were selected on the basis of the different characters and degrees of response of the dark chromatophores of the body proper upon which the extracts were tested simultaneously. The basis for

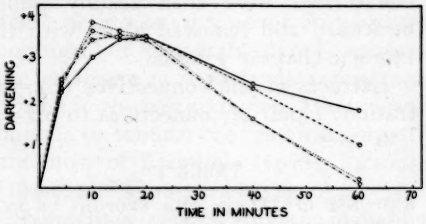

FIG. 1.—The relation between the degree of darkening of the telson and uropods of eyestalkless *Crago* and the time following an injection of connectives extract (equivalent to 0.15 pairs of connectives). Each curve represents the average of about seven experiments. Simultaneous influences upon body coloration are seen in similarly characterized plots in Fig. 3.

the four groups will be described later. The results are graphically represented in Figure 1.

Various concentrations of extracts of the connectives, ranging from 12 to 0.5 connectives per milliliter, were tested. In this latter series there was found a gradual reduction in the effectiveness of the extract in its telson- and uropod-darkening action in passing from the more concentrated to the more diluted solutions.

The influence of the extracts upon the chromatophores of the remainder of the body was far less uniform and predictable. The response involved lightening alone or lightening followed by darkening. Before injecting a given eyestalkless animal this animal was always closely matched, in shade and coloration, with another eyestalkless animal; and the latter was kept as a control. The degree of lightening, relative to the control, at any given moment following injection was indicated by a figure between 0 and −4; the degree of darkening, with respect to the control, by a figure between 0 and +4. It was possible to arrange all the results to form a more or less continuous series. Figure 2 shows the range of variation of response of the dark pigment of the body observed in the twenty-seven experiments. In some of the experiments there was a strong lightening of the body lasting from 40 to 70 minutes. This is seen in Table 2, column 4. In other cases there was an initial lightening of the body lasting from 15 to 25 minutes, followed by a transitory and characteristic darkening, which progressed to a state nearly as dark as, or perceptibly darker than, the initial condition but lacked the mottling so characteristic of eyestalkless specimens. Columns 2 and 3 of Table 2 show such responses. In Plate I, *A* and *B* show the results of an injection experiment after 5 and 30 minutes,

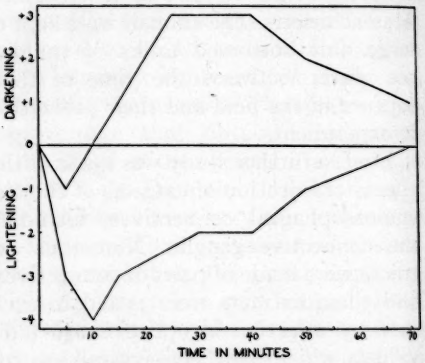

FIG. 2.—The range of responses of bodies of eyestalkless *Crago* to injection of sea-water extracts of circumoesophageal connectives (dosage the equivalent of 0.15 of one pair of connectives). Twenty-seven individual experiments showed all responses lying between these extremes.

respectively. In each figure the injected animal is on the right; on the left there is an eyestalkless animal which matched very closely the coloration of the injected animal at the time of injection. In still

other cases there was a much stronger darkening of the body which followed upon a relatively brief period of lightening. Column 1 of Table 2 gives the average of seven such cases. In these latter experiments the darkening of the body which followed the initial lightening phase usually possessed certain noticeable characteristics, including, in addition to the lack of mottling, a more rapid darkening of the posterior extremity and lateral aspects of the abdomen. The time of return to the initial intermediate and mottled coloration for all experiments coincided very closely with the time of lightening of the telson and uropods.

TABLE 2

RESPONSE OF BODIES OF EYESTALKLESS ANIMALS TO INJECTION OF EXTRACT OF CIRCUMOESOPHAGEAL CONNECTIVES

Concentration: 1 pair of connectives per 0.2 ml. sea water; dosage: 0.03 ml. per animal. The signs + or − indicate darkening or lightening, respectively; and the numbers 0–4, the degree of either, compared with similar parts of control eyestalkless animals.

Time (Min.)	(1) Av. of 7	(2) Av. of 7	(3) Av. of 6	(4) Av. of 7
0.....	0.0	0.0	0.0	0.0
5.....	−2.2	−1.8	−2.3	−2.4
10.....	−1.0	−0.8	−1.8	−2.6
15.....	+0.6	−0.2	−0.3	−1.9
20.....	+1.4	+0.3	−0.1	−1.0
40.....	+1.7	+0.6	0.0	−0.6
60.....	+0.2	0.0	0.0	0.0

The results obtained in the four groups are depicted graphically in Figure 3. There is roughly an inverse correlation between the degree of lightening shown in the first 5 or 10 minutes and the degree of the characteristic secondary darkening. By comparing these results with those observed for telson and uropods (Fig. 2), it is seen that the cases showing more body-darkening are also the same cases in which there is, on the average, a greater and longer-lasting darkening of the telson and uropods.

The same general series of types of response was also observed in numerous experiments in which the eye stubs of eyestalkless animals were stimulated by a hot needle or electrically with a faradic current. In general, weaker stimuli tended to give predominantly the body-darkening reaction, while stronger ones appeared, in general, to increase the duration of the lightening phase. In these experiments body-darkening sometimes occurred directly upon eye-stub stimulation without any significant lightening phase, and under these circumstances the body usually became very dark in 25–30 minutes and remained dark for 1 to 2 hours.

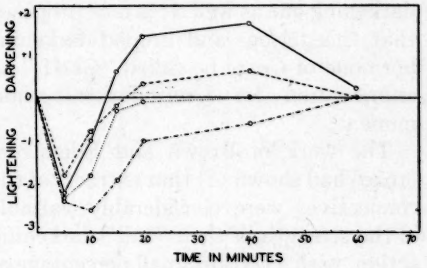

FIG. 3.—The relation between the lightening or darkening of the body and the time following injection of connectives extract (equivalent to 0.15 pairs of connectives). The effects of twenty-seven extract samples were averaged in groups of about seven on the basis of the relative degrees of body-lightening or -darkening. Simultaneous influences of these extracts upon telson- and uropod-darkening were seen in Fig. 2.

In one instance an eyestalkless animal, with eye stubs stimulated electrically, darkened greatly in 25 minutes. At this point there was injected 0.03 ml. of extract of *Pagurus* (hermit crab) connectives in 0.2 ml. sea water. These latter organs had been shown to possess a body-lightening action but no influence

upon coloration of the telson and uropods of *Crago*. The body, but not the telson and uropods, blanched completely in 3–5 minutes; and then, after about 15–20 minutes, as the effect of the injected substance apparently wore off, the body again darkened, remaining dark as long as did the telson and uropods.

All of the foregoing observations are entirely compatible with, but do not conclusively prove, the hypothesis that the telson- and uropod-darkening principle is, under certain circumstances, a body-darkening one as well. It is now proposed that this telson- and uropod-darkening hormone of *Crago* be called "CDH," an abbreviation for *Crago*-darkening hormone.

The work of Brown and Ederstrom (1940) had shown (1) that extracts of the connectives were considerably variable in the strength of their "tail"-darkening action, with a certain small percentage of the extracts showing no activity, and (2) that the point of highest activity appeared to lie not in the connective ganglion, where one might reasonably expect it, in view of the presence there of large numbers of cell bodies, but rather in that region of a connective just posterior to this ganglion. These observations appeared to indicate either that the connectives of *Crago* varied considerably from specimen to specimen in their CDH content, despite similar history of the animals, as far as could be determined, or that the actual source of CDH was not always being fully removed for extraction along with the connectives with which they were perhaps only closely associated. The resolution of these questions called for more critical localization experiments. It was also hoped that more rigorous localization of the source of CDH might perhaps permit one to obtain an extract containing this hormone but not the body-lightening one with which it had hitherto always been mixed.

Initial exploratory experiments confirmed entirely the conclusion that the greater part of the CDH activity of the connectives lie posterior to the ganglion rather than in it. Parts *A* and *C* (Fig. 4) showed little or no activity; part *B* contained nearly all of it. It was also noticed that there was a rough, direct correlation between the length of the tritocerebral commissure remaining attached to part *B* of the connective and the chromatophorotropic activity of the extract made from the piece. The tritocerebral commissure is a very constant structural feature of the crustacean nervous system and includes a nerve tract passing from the connective ganglion of one side, posteriorly down the medial surface of the connective for a short distance, and then proceeding to the contralateral connective around the posterior side of the oesophagus. This relationship is diagrammatically represented in Figure 4. Upon the tritocerebral commissure are two slight enlargements, each one approximately a third of the distance along the commissure and appearing to contain cell bodies.

The tritocerebral commissures were next assayed for CDH content. These commissures were readily removed intact under a binocular dissecting microscope in the following manner: The dorsal aspect of the carapace was removed, and the animal eviscerated as in the earlier experiments. In this case, in evisceration, the oesophagus was grasped firmly with forceps and torn away at the mouth region. Next, the circumoesophageal connectives were severed at their point of junction with the thoracic chain of ganglia. With fine jewelers' forceps the nerves leading from the brain to the anterior appendages and sense organs were

cut and the brain lifted up and backward in such a manner as to permit the posterior ends of the connectives to be drawn forward through the skeletal passageway and upward, free from the body. This technique usually left the tritocerebral commissures undamaged and still attached at both ends to the connectives. Approximately once in five attempts the commissures would be broken, in which case the preparation was discarded. This wastage was considered very small, in view of the fact that the foregoing extirpation could usually be accomplished in about 1 minute. The connective-commissure complex was then placed in a Syracuse watch glass in sea water, and with suitable magnification the commissure could be cut free from the connective with a sharp scalpel at the point of its departure, as indicated by the broken line, *E*, in Figure 4. Extracts of single tritocerebral commissures in 0.2-ml. portions of sea water and extracts of the residual parts of the two connectives similarly in 0.2 ml. were prepared. Each extract was assayed upon two eyestalkless animals, each of which received a dosage of 0.03 ml. In every experiment the extracts of the tritocerebral commissures produced very strong darkening of the telson and uropods of the injected animals. The extracts of the remaining connectives always gave weaker but highly variable responses, ranging from no significant darkening in some instances to moderately strong darkening in others. Plate I, *C*, shows the result of a typical experiment with two such animals injected about 8 minutes before the photograph was taken. The animal on the right received extract of a tritocerebral commissure; the one on the left received extract of the remaining connective.

Extraction of isolated parts of a tritocerebral commissure was attempted in one experiment. The two small enlargements on the commissure, together with the intervening tissue, were separated from the lateral portions, as indicated by the broken line in Figure 4 separating *F* from *G*. Portions *F* and *G* were extracted separately in 0.2-ml. portions of sea water, and these extracts were compared in their activity with one another and with extracts of the remaining connectives. All three extracts produced darkening of the telson and uropods of test animals. The extract of *G* produced in the test animals a maximal "tail"-darkening which lasted for more than an hour. Simultaneously there was strong body-lightening, followed in about half an hour by slight darkening. The extracts of *F* and of the remaining connectives gave a weaker "tail"-darkening and, simultaneously, a slight body-darkening; these were evident at 15 minutes.

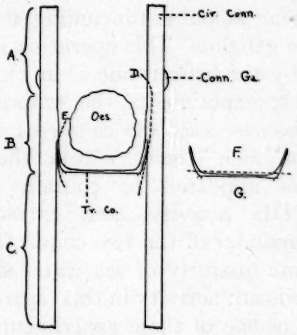

FIG. 4.—A diagram of the circumoesophageal complex of *Crago* illustrating the portions experimentally investigated for endocrine activity in this report. Regarding *A*, *B*, *C*, *D*, *E*, *F*, *G*, see text. *Cir. Conn.*, circumoesophageal connectives; *Conn. Ga.*, connective ganglion; *Oes.*, position of the oesophagus; *Tr. Co.*, tritocerebral commissure.

A final localization experiment was conducted to learn in what portion of the connectives proper was the maximum

activity after removal of the tritocerebral commissure. In several experiments the tritocerebral commissure was separated from the connective by a longitudinal cut aimed at removing from the connective that portion which appeared to be simply a continuation of the commissural tract toward the connective ganglion. The median aspect of the connective thus removed with the commissure included only a region from the point of the apparent origin of the latter, anteriorly as far as, but not including, the connective ganglion. This operation is indicated by the broken line *D* in Figure 4. In these experiments the tritocerebral commissures and the apparent extension of their tracts within the connectives appeared to contain all of the CDH activity, and extracts of the remainder of the two connectives in the same quantity of sea water showed no significant activity in this regard. The results of one of these experiments is illustrated in Plate I, *D*. Twelve minutes before the photograph was taken, the two upper animals were injected with an extract of tritocerebral commissure and the adjacent connective region, while the two lower animals received an injection of extract of the remaining connectives, including the connective ganglia. It thus appears that the CDH activity of the connectives proper, earlier shown to be most abundant in the region posterior to the ganglion, actually lies along the median face of the connective between the ganglion and the point at which the tritocerebral commissure becomes free.

Microscopic examination of a freshly dissected commissure in sea water showed a relatively high concentration of a bluish-green particulate substance lying between the outer nerve sheath and the central nerve fibers. The concentration appeared greatest near the two enlarged portions of the commissure.

GENERAL CONCLUSIONS AND SUMMARY

The dark chromatophores of eyestalkless *Crago* respond to injection of extracts of the circumoesophageal connectives in such a manner as to support a hypothesis that this region of the central nervous system produces two chromatophorotropic principles. One of these two hormones is believed to act to lighten all of the body except the telson and uropods, while the other is believed to darken the telson and uropods and, in the absence of the former, to darken the rest of the body as well.

This hypothesis is also supported by the character of the responses which follow stimulation of the stubs of the optic nerves of eyestalkless specimens.

Localization experiments show that the point of origin of the darkening hormone, CDH, in *Crago* is in the tritocerebral commissure and that all significant activity of the nervous system in this regard is restricted to this commissure, together with its immediate continuation within the connectives.

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PLATE I

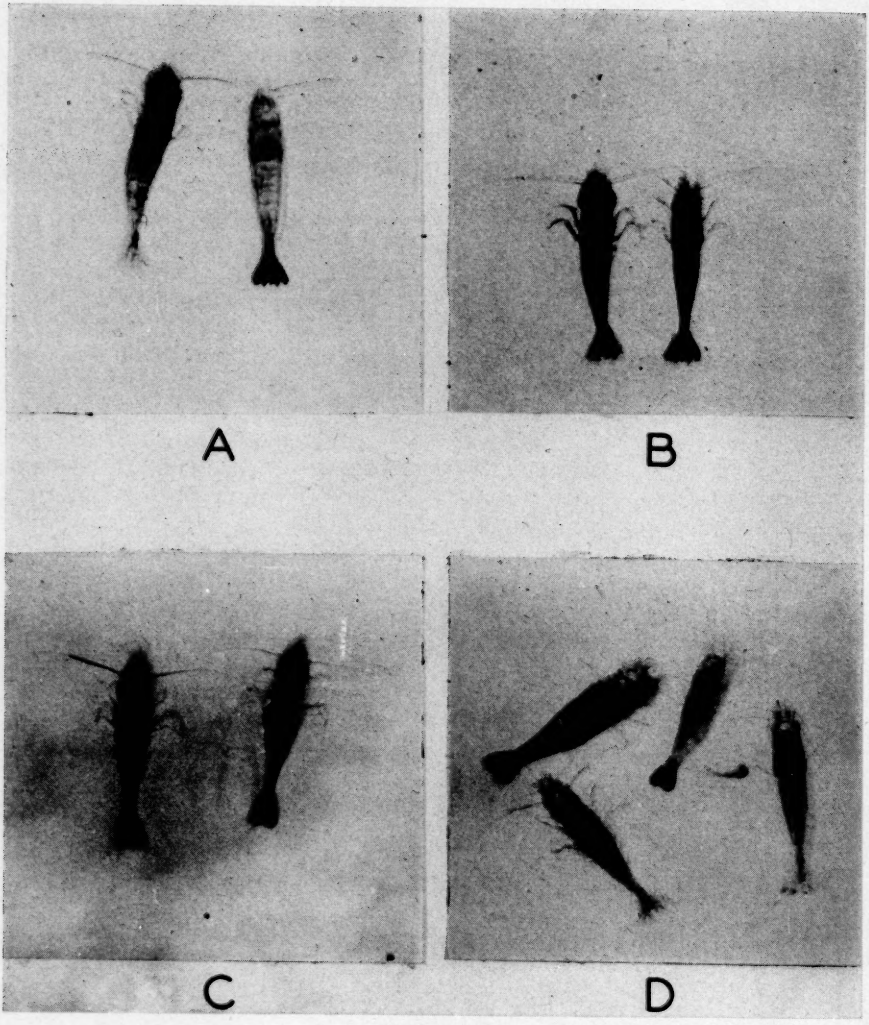

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PLATE I

A AND *B*.—The shrimps on the right in these figures were injected with connectives extract 5 and 30 minutes, respectively, before being photographed. Those on the left were untreated controls which just matched in coloration the treated animals at the beginning of the experiment.

C.—Eight minutes before photographing, the shrimp on the right was injected with an extract that was the equivalent of 0.15 of a tritocerebral commissure. The shrimp on the left received a similar dosage of extract of the remaining connectives.

D.—Twelve minutes preceding the taking of the photographs, the two upper shrimp received an injection of an extract of a tritocerebral commissure, including the adjacent face of the connectives (the equivalent of 0.15 of one organ). The two lower specimens received extract of the remainder of the connective complex, including the connective ganglia, in the same concentration.

THE NATURE OF THE GROWTH-SUBSTANCE PRODUCED BY CHILOMONAS PARAMECIUM[1]

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THE results obtained in observations made by various investigators on a considerable number of different species of protozoa indicate that probably all protozoa produce a substance which accelerates growth in them (Robertson, 1922, 1923, 1924; Yocum, 1928; Petersen, 1929; Dimitrowa, 1932; Mast and Pace, 1938; Sweet, 1939; Kidder, 1941; Marbarger, 1943).

Very little is known concerning the nature of this substance. Mast and Pace (1938) conclude that, while the growth-substance produced by *Chilomonas paramecium* greatly accelerates growth in low concentration, it retards growth and is lethal in high concentration, and they maintain that it is destroyed by exposure to 100° C. for 90 minutes. Marbarger (1943) reached similar conclusions in reference to *Colpidium striatum* Stokes. Kidder (1941) contends, however, that in *Tetrahymena geleii* Furgason[2] two distinct substances are produced, one which accelerates and one which retards growth. The following pages contain further evidence concerning the effect and the nature of the growth-substance produced by *C. paramecium*.

[1] These investigations were aided by a grant from the American Philosophical Society.

[2] See n. 10, p. 232.

MATERIAL

The chilomonads used were obtained from pure cultures started nearly 12 years ago and maintained in a sterile solution of sodium acetate, ammonium chloride, magnesium sulfate, potassium phosphate, calcium chloride and redistilled water (Mast and Pace, 1938). This solution will be referred to as the acetate-ammonium or ac-am solution. The chemicals used were Kahlbaum's purest, and the water was triple distilled in pyrex glass vessels. All the glassware used was made of pyrex glass. Sterile cultures were used exclusively in all the following experiments.

FURTHER EVIDENCE CONCERNING THE EFFECT OF THE GROWTH-SUBSTANCE PRODUCED BY *Chilomonas paramecium*

Hall (1941) contends that the evidence presented by Mast and Pace (1938) concerning the growth-substance produced by *C. paramecium* does not warrant their conclusions. The following experiments concern these conclusions.

EXPERIMENT I

Four sacks, made of chemically clean Cellophane membranes,[3] were fastened to glass tubes and suspended in four 125-cc. Erlenmeyer flasks, each containing 60 cc. acetate-ammonium solution (Fig. 1). Then 10 cc. acetate-ammonium solution were put into each sack and the four preparations sterilized by subjecting them to 70° C. for 1 hour on each of 3 successive days. The flasks were now numbered, and 0.1 cc. fluid from a flourishing sterile chilomonad culture (approximately 10,000 chilomonads per cubic centimeter) added to the fluid in the sack in No. 1; 0.6 cc. to the fluid in the flask in No. 2; 0.6 cc. to the fluid in the flask and 0.1 cc. to the fluid in the sack in No. 3; and none to the fluids in the flask or the sack in No. 4. Then the four preparations were put into the temperature chamber at 24° C. and left until the chilomonads had died. The length of life in each preparation was recorded and the experiment repeated.

[3] Du Pont Cellophane, No. 600.

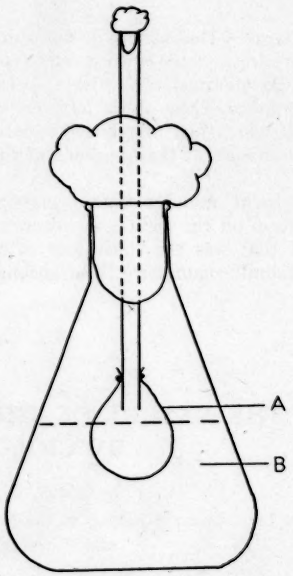

FIG. 1.—Diagram of the apparatus used in experiments on the size of the molecules of the growth-substance produced by *Chilomonas*. *A*, sack made of Du Pont Cellophane, No. 600; *B*, Erlenmeyer flask.

It was found that in No. 1, with approximately 100 chilomonads per cubic centimeter in the sack at the beginning and none in the flask, all died in from 8 to 10 days; that in No. 2, with approximately 100 chilomonads per cubic centimeter in the flask and none in the sack, all died in from 4 to 6 days; and that in No. 3, with approximately 100 chilomonads per cubic centimeter in the sack and in the flask, all died in from 4 to 5 days. If the death of the chilomonads was due to

excessive accumulation of growth-substance, as the evidence presented by Mast and Pace (1938) strongly indicates, the fact that the chilomonads in the sack in No. 1 with no chilomonads in the flask lived twice as long as those in the sack in No. 3 with chilomonads in the flasks shows that some of the growth-substance produced in the sack in No. 1 passed out through the Cellophane membrane and consequently delayed the lethal concentration of the solution in the sack. This conclusion is in harmony with the fact that the chilomonads in the sack died sooner in No. 3 than in No 1, for in No. 3 the concentration of growth-substance was nearly the same in the flask as in the sack, so that but little could pass out of the sack, resulting in more rapid lethal concentration in No. 3 than in No. 1.

EXPERIMENT II

This experiment was the same as the preceding, except that the chilomonads were not left in the sacks and flasks until they died but were removed and counted 4 days after the preparations were made. The results obtained are presented in Table 1.

This table shows that the chilomonads in the sack in No. 1 with no chilomonads in the flask reproduced nearly ten times as rapidly as those in the sack in No. 3 with chilomonads in the flask.

This great difference in rate of reproduction is intelligible on the assumption that some of the growth-substance produced in the sack in No. 1 passed out through the Cellophane membrane and consequently kept the concentration so low that it accelerated growth and that in the sack in No. 3, since but little could pass out, it became so concentrated that it retarded growth.

The results presented in Table 1 therefore support the conclusions reached above that the growth-substance produced by *Chilomonas* can pass through the Cellophane membrane and also the contention of Mast and Pace that *Chilomonas* produces a substance which accelerates growth in low, and retards it in high, concentrations and ultimately kills the chilomonads.

TABLE 1

DIFFUSION OF GROWTH-SUBSTANCE THROUGH A CELLOPHANE MEMBRANE AS INDICATED BY ITS EFFECT ON RATE OF REPRODUCTION. 1, 2, 3, PREPARATIONS, EACH CONSISTING OF A CELLOPHANE SACK CONTAINING AC-AM SOLUTION SUSPENDED IN AC-AM SOLUTION IN A FLASK (FIG. 1)

Designation of preparations	Number of Chilomonads per Cubic Centimeter			
	Put into Sack	In Sack after 4 Days	Put into Flask	In Flask after 4 Days
	Experiment IIA			
1	100	105,600	0	0
2	0	0	100	10,500
3	100	10,800	100	9,600
	Experiment IIB			
1	100	89,000	0	0
2	0	0	100	11,000
3	100	9,500	100	11,000

EXPERIMENT III

This experiment was performed in the same way as Experiment II, except that after the chilomonads had been in the sacks and flasks 4 days or less some fluid was drawn off and the rate of growth in it ascertained. This was done by measuring with the method used by Mast and Pace (1938), Marbarger

(1943), and Pace (1944) the time required for the first generation[4] of chilomonads in it. All the chilomonads used in these measurements were the same in age and physiological state, and all were subjected to the same conditions; for all were obtained from a sterile, young, vigorous culture, and each specimen was taken immediately after fission, passed successively through four 0.1-cc. portions of fresh ac-am solution, and then put into 0.025 cc. fluid (taken from the sacks or flasks) in a depression on a slide in a Petri dish. Sixteen chilomonads were used in each test, and all were kept in the temperature chamber at 24° C. The results obtained are presented in Table 2.

TABLE 2

DIFFUSION OF GROWTH-SUBSTANCES THROUGH A CELLOPHANE MEMBRANE AS INDICATED BY ITS EFFECT ON RATE OF GROWTH: 1, CHILOMONADS IN CELLOPHANE SACK BUT NONE IN FLASK; 2, CHILOMONADS IN FLASK BUT NONE IN SACK; 4, NO CHILOMONADS IN SACK OR FLASK. A, B, AND C. SOLUTION DRAWN OFF AFTER 4 DAYS AND TESTED FOR RATE OF GROWTH; D, SAME AFTER 10 HOURS, SIXTEEN ISOLATED CHILOMONADS WERE USED IN EACH TEST

Solution Tested	Average Time in Hours Required for First Generation in:				
	Expt. IIIA	Expt. IIIB	Expt. IIIC	Total Average A, B, C	Expt. IIID
Fluid taken from flask (1).....	7.25±0.18*	7.25±0.12	7.60±0.21	7.36±0.17	5.88±0.16
Fluid taken from sack (2).....	9.54±0.24	9.74±0.37	9.62±0.28	9.63±0.30	5.41±0.26
Fluid taken from flask (4).....	6.41±0.15	6.22±0.13	6.25±0.09	6.29±0.12	6.88±0.14
Fresh ac-am solution.....	6.40±0.09	6.20±0.20	6.34±0.13	6.31±0.14	6.62±0.10

* Standard error.

This table shows that the rate of growth of *Chilomonas* was the same in the ac-am solution in which a Cellophane sack had been suspended as in that which had not been in contact with Cellophane. This shows that the Cellophane sacks used in the experiment are not involved in the results obtained. The table also shows that growth was much slower in the solution taken from the flask in No. 1 after chilomonads had developed for 4 days in the Cellophane sack suspended in it than in the solution taken from the flask in No. 4 with no chilomonads in the sack, but that it was probably considerably slower in the latter than in the solution taken from the flask in No. 1 after chilomonads had developed for only 10 hours in the sack, the average time required for one generation in the three solutions having been, respectively, 7.36, 6.29, and 5.88 hours.[5]

[4] The time from the separation of two individuals formed by fission until they, in turn, have divided, i.e., the time required for the development and division of a chilomonad. This will be referred to as "generation-time."

[5] Our tables contain standard errors, and we have calculated the values of t and P for all the results used in reaching the conclusions presented. These values show that all the deductions based upon our results are unquestionably valid, with the possible exception of the following:

1. P. 226, *re* Table 2, 6.29 ± .12 vs. 5.88 ± .16; $t = 2.05$, $P = <.05$.
2. P. 230, *re* Table 5, 6.78 ± .01 vs. 6.55 ± .09; $t = 2.54$, $P = <.015$.
3. P. 232, *re* Table 6, 10.15 ± .43 vs. 9.25 ± .22; $t = 1.87$, $P = .075$.

These values are significant to us. Others are, of course, at liberty to dissent. Many would doubtless question No. 3. The experiment referred to in No. 3 was, however, repeated (see Table 6), and the results obtained are 10.72 ± .37 vs. 9.00 ± .23; $t = 3.95$; $P = <.001$, i.e., approximately .0007.

The results presented show, therefore, that some substance produced by the chilomonads passed through the Cellophane membrane, and they indicate that in low concentration this substance accelerated, and in high retarded, growth. That is, the results very strongly support the conclusion reached above.

Hall (1941) maintains that the conclusions reached by Mast and Pace (1938) are not valid, owing to the inadequate methods used. He says: "Mast and Pace did not wash their flagellates thoroughly before inoculation of experimental cultures, and their method would have insured transfer of conditioned medium." In the transfers referred to by Hall, each chilomonad was, as clearly stated, passed successively through four 0.1-cc. portions of fresh sterile ac-am solution, and in each transfer not more than 0.5 cmm. of the solution was transferred with the chilomonad. It is obvious, therefore, that the dilution of the culture fluid transferred to the test solutions was very great. Calculations show, indeed, that there was less than 1 part in 1.5 billion. Moreover, the concentration of growth-substances in the culture fluid was low, so that, after this was diluted more than a billion times, it is certainly not likely to have had any measurable effect; and, since the same method was used in the present experiments, the results obtained would seem to be reliable.

THE SIZE OF THE MOLECULE OF GROWTH-SUBSTANCE PRODUCED BY *C. paramecium*

The results presented above demonstrate that the growth-substance produced by *C. paramecium* readily passes through the membrane used. Its molecules must therefore be smaller than the pores in this membrane. The size of these pores was measured as follows:

A Cellophane disk[6] which had been in redistilled water 24 hours, was clamped in the holders in each of two "ultrafilters" (Allisbaugh and Hyde, 1935). The cups in the filters were filled with redistilled water, and each apparatus connected with a vacuum pump and a mercury manometer, care being taken to make the whole system airtight. Then the pressure was reduced to the desired level and held there by closing the connection between the filters and the pump, and the amount of water that passed through the membranes in a given time measured. This was repeated several times with each of several membranes, some of which differed in size. The results obtained were substituted in the following equation (Cox and Hyde, 1932), and the diameter of the pores in the membrane calculated:

$$r = L\sqrt{\frac{8\eta q}{m}},$$

in which r is the radius of the pores in centimeters; L, the length of the pores (thickness of the membrane[7]); 8, a constant; η, the coefficient of viscocity of the fluid in cm./gm./sec. (η for water at 20° C. = 0.01005); q, the amount of fluid in cubic centimeters that passes through 1 sq. cm. of membrane per second under pressure of 1 dyne per square centimeter; and m, the amount of fluid in the membrane in grams per square centimeter.

Taking the results of these two experiments together, we think the conclusion we reached is strongly supported.

[6] The disks were made of Cellophane, No. 600, i.e., Cellophane membrane like that used in the sacks described above.

[7] This was ascertained by measuring sections of the membrane with an ocular micrometer.

The results of the calculations are given in Table 3. These results indicate that the pores in the Cellophane membrane in the sacks used in the preceding experiments are approximately 6 mμ in diameter. The molecules of the growth-substance produced by *Chilomonas* are therefore less than 6 mμ in diameter.

Further evidence concerning the size of these molecules was obtained in observations on the permeability of the Cellophane membrane to hemoglobin, albumin and globulin from the blood of the rat, and sucrose and glucose. It was found that, whereas the growth-substance (as stated above) passes through the membrane readily, the hemoglobin, albumin, and globulin do not pass through it, and sucrose and glucose, especially the former, only *very* slowly. The molecules of the growth-substance are therefore smaller than those of the hemoglobin and the albumin or the globulin in the blood of the rat, and probably smaller than those of sucrose or glucose.

TABLE 3

SIZE OF THE PORES IN THE CELLOPHANE MEMBRANE USED IN THE PRECEDING EXPERIMENTS. IN A, B, AND D THE TWO MEMBRANES USED WERE EQUAL IN SIZE. IN B, THERE WAS A LEAK IN ONE; HENCE ONLY ONE RESULT IS GIVEN

Area of membrane	Calculated Diameter of Pores in mμ			
	Test A	Test B	Test C	Test D
1 cm²	6.4 7.8	7.8	5.6	.....
2 cm²	.....	.....	5.0	.....
6 cm²	.....	.....	.....	5.96 6.00
Total average	6.36			

THE EFFECT OF AGING ON GROWTH-SUBSTANCE PRODUCED BY *C. paramecium*

It was observed at various times during the past several years that, in cultures of *Chilomonas* which had been left in the temperature chamber after they had died out, occasionally specimens again appeared in a few weeks; became fairly abundant (though never so abundant as they had been); and then soon disappeared again. A few of the chilomonads in the culture in which all appeared to have died doubtless remained alive, and the development which appeared later doubtless resulted from reproduction of these. The fact that these stragglers began to grow and multiply after having been quiescent for several weeks shows that either they or the medium in which they lived must have changed during this time. The following experiments concern this problem.

Several sterile cultures of chilomonads (started by adding to 1.2 cc. ac-am solution in each of several Columbia dishes in Petri dishes, 0.1 cc. from a flourishing culture containing approximately 10,000 chilomonads per cubic centimeter) were left until all the chilomonads had died and settled to the bottom and the growth-substance had become maximum in concentration.[8] Then most of the solution in all the dishes was drawn off with a fine pipet and half put into each of two small test-

[8] Robbins and Ma (1942) demonstrated that cotton batting contains biotin, pyridoxine, and thiamine. This has led some to maintain that culture fluid in flasks closed with cotton plugs becomes contaminated. Robbins and Ma did not find any evidence of this, unless water passed through the plug into the flask. We made various tests concerning this and also found no evidence of contamination if the cotton in the plugs did not become wet. In cultures in Columbia dishes in Petri dishes, the possibility of such contamination is obviously avoided.

tubes. The rate of growth in the solution in one of these testtubes was now ascertained by means of the method used in preceding experiments. The other testtube was put into the temperature chamber at 24° C. and left 3 weeks (water lost by evaporation being replaced from time to time by adding redistilled water); then the rate of growth of chilomonads in the solution in the tube was ascertained as described above. This experiment was repeated. The results obtained are given in Table 4.

TABLE 4

THE EFFECT OF "AGING" ON THE GROWTH-SUBSTANCE PRODUCED BY *Chilomonas*. A, 32 ISOLATED CHILOMONADS USED IN EACH TEST; B, 48 ISOLATED CHILOMONADS USED IN EACH TEST

SOLUTION FROM A CULTURE	AVERAGE TIME IN HOURS REQUIRED FOR FIRST GENERATION IN:	
	Expt. A	Expt. B
Immediately after all chilomonads had died	9.78±0.35	10.35±0.41
Three weeks after all chilomonads had died	7.25±0.28	7.50±0.32

Table 4 shows that the rate of growth in the culture fluid in which the chilomonads had died out was much lower immediately after the chilomonads had died than it was 3 weeks later; and comparison of Tables 4 and 5 shows that the rate of growth in the older culture fluid was practically the same as in fresh culture fluid, i.e., ac-am solution in which no chilomonads had grown. This shows that fluid in which a culture of chilomonads has died out changes with age at room temperature and that if the reduced rate of growth in old culture fluid is due to excessive growth-substance, as Mast and Pace (1938) contend, this substance gradually disintegrates[9] at room temperature. It is, therefore, highly probable that the observed spontaneous rejuvenation of cultures, several weeks after they appear to have died out, is due to disintegration of growth-substance, not to adjustment of the few surviving chilomonads to the toxic concentration of this substance.

THE EFFECT OF DRYING ON THE GROWTH-SUBSTANCE PRODUCED BY *C. paramecium*

EXPERIMENT I

Several cultures of chilomonads were started and treated as described above. After all the organisms had died and the growth-substance had become maximum in concentration, most of the solution in all the dishes was removed and divided into two equal parts. One part was put into a small testtube and left in the temperature chamber at 24° C. The other part was put into Columbia dishes in Petri dishes in a desiccator connected with a vacuum pump by means of a tube which contained a water-trap. The trap was immersed in a mixture of solid carbon dioxide and 95 per cent alcohol, and the pressure in the desiccator lowered to about 50 mm. Hg. This caused the water in the dish to evaporate rapidly without any increase in tempera-

[9] It has been suggested that the observed decrease in rate of growth may have been due to evaporation or adsorption rather than to disintegration of growth-substance. We have, however, obtained similar results in cultures kept in closed flasks and in cultures kept in a damp chamber in which there was no evaporation; and if adsorption were involved (this being a rapid process), it would have required only a short time instead of 3 weeks to produce the observed effect, and the generation-time in old culture fluid kept for 3 weeks at 0° and 24°, respectively, should have been the same, instead of 9.15 hours for the one and only 7.37 for the other (Table 7).

ture. After all the water had evaporated, an equal amount of redistilled water was added to the residue, after which the same amount of fresh ac-am solution was put into the desiccator and evaporated and an equal amount of redistilled water added to the residue. Then the rate of growth of *Chilomonas* in these two solutions—that in the testtube and that in the fresh ac-am solution—was simultaneously ascertained by means of the method used in the preceding experiments.

added; E, same as D except that the original fluid was obtained from a medium-aged culture (200 chilomonads per 1.2 cc., left 24 hours). The growth-substance in this fluid was therefore moderate in concentration.

The results obtained are presented in Table 5. This table shows that evaporation of fresh ac-am solution does not significantly affect the rate of growth in it, the total average time required for a generation having been 7.41 hours in fresh ac-am solution and 7.34 hours in

TABLE 5

THE EFFECTS OF DRYING ON GROWTH-SUBSTANCE PRODUCED BY *Chilomonas*. IN A AND B, FLUID TESTED WAS FROM OLD CULTURES CONTAINING GROWTH-SUBSTANCE IN HIGH CONCENTRATION; C AND D, FLUID FROM YOUNG CULTURES CONTAINING GROWTH-SUBSTANCE IN LOW CONCENTRATION; E, FLUID FROM MEDIUM-AGED CULTURE CONTAINING GROWTH-SUBSTANCE IN MODERATE CONCENTRATION. THIRTY-TWO ISOLATED CHILOMONADS WERE USED IN EACH TEST

SOLUTIONS	AVERAGE TIME IN HOURS REQUIRED FOR FIRST GENERATION IN:				
	Expt. A	Expt. B	Expt. C	Expt. D	Expt. E
Fresh ac-am	7.10±0.18	6.63±0.21	7.43±0.10	7.92±0.09	7.07±0.32
Fresh ac-am evaporated	7.23±0.15	6.59±0.23	7.26±0.09	7.72±0.15	6.82±0.30
Fluid from cultures	9.89±0.21	9.56±0.28	6.58±0.08	6.55±0.09	6.15±0.27
Fluid from cultures, evaporated	8.62±0.18	6.38±0.19	6.68±0.08	6.78±0.01	6.27±0.23

This experiment (designated A) was repeated four times (B, C, D, E) with the following modifications: B, same as A except that the residue of the evaporated culture fluid was left exposed to air in the desiccator for 24 hours before the redistilled water was added; C, same as A except that the original fluid was obtained from a young culture (500 chilomonads per cubic centimeter of ac-am solution, 4 hours) instead of from one in which all the chilomonads had died (the concentration of growth-substance was therefore low in this fluid); D, same as C except that the residue of the evaporated culture fluid was left exposed to air in the desiccator for 12 hours before water was this solution after evaporation and addition of redistilled water.

By referring to Experiments A and B in Table 5 it will be seen that the time required for one generation was roughly 6.8 hours in fresh ac-am solution; 9.7 hours in old culture fluid; 8.6 hours in old culture fluid after evaporation and addition of redistilled water; and 6.4 hours in old culture fluid after evaporation, exposure of the residue to air for 24 hours, and addition of redistilled water. This shows that the rate of growth in old culture fluid is much lower than it is in fresh culture fluid and that drying and exposure to air, especially the latter, greatly reduce its retarding action.

Consequently, if this retarding action is due to growth-substance in excessive concentration, as Mast and Pace (1938) maintain, it shows that the growth-substance produced by *Chilomonas* is destroyed by drying and exposure to air.

Reference to C and D (Table 5) shows that the time required for one generation was roughly 7.6 hours in fresh culture fluid and 6.6 hours in fluid from the young cultures, i.e., that the rate of growth was much higher in the fluid from the young cultures than in fresh culture fluid. It also shows that evaporation of fluid from the young culture (C) caused only a very slight decrease in rate of growth, if any (the average increase in generation-time being only from $6.58 \pm .08$ to $6.68 \pm .08$ hours), but that evaporation and exposure of the residue to air for 12 hours caused a definite decrease in rate of growth (i.e., an increase in generation-time from $6.55 \pm .09$ to $6.78 \pm .01$).

Table 5 shows, however, that the rate of growth in fluid from the young culture (D) after evaporation and exposure of the residue to air was considerably higher than in fresh culture fluid, whereas in fluid from the old culture (B) after evaporation and exposure it was practically the same as in fresh culture fluid. This difference was probably due to the fact that the latter was exposed twice as long as the former. By comparing A with B and C with D, it will be seen, however, that, in reference to both old and young cultures, exposure of the residue to air had a much greater effect on the growth-substance than did evaporation. This seems to show that this substance is not volatile, and it suggests that oxidation is involved in its destruction.

Table 5 (E) indicates that in the fluid from the medium-aged culture, the rate of growth was nearly, if not quite, the same as in fresh culture fluid and that evaporation had but little, if any, effect on it. This is precisely what would be expected if, as Mast and Pace maintain, the concentration of growth-substance increases with the age of the culture, and, as it increases from zero, the rate of growth increases to a maximum and then decreases to zero.

THE EFFECT OF OXYGEN ON GROWTH-SUBSTANCE PRODUCED BY *C. paramecium*

Culture fluid containing chilomonad growth-substance was obtained as in the preceding experiment. One-third of this fluid was put into each of three small testtubes, and pure oxygen and nitrogen gas bubbled, respectively, through two of them for 5 hours. Then the rate of growth of *Chilomonas* in each of the three solutions and in fresh ac-am solution was ascertained simultaneously as described above. This experiment was repeated. The results obtained are recorded in Table 6.

This table shows that the rate of growth was much lower in the old culture fluid than in the fresh but that it was considerably higher in the old fluid which had been subjected to pure oxygen than in the old untreated fluid and only very slightly, if any, higher in the old fluid which had been subjected to pure nitrogen. This shows that oxygen neutralizes the retarding action of old culture fluid, and, if this action is due to the presence of an excessive amount of growth-substance, it indicates that oxygen destroys this substance, i.e., that the disintegration of growth-substance is, at least in part, due to oxidation.

THE EFFECT OF TEMPERATURE ON THE GROWTH-SUBSTANCE PRODUCED BY *C. paramecium*

Mast and Pace (1938) imply that chilomonad growth-substance in maximum concentration disintegrates completely in 90 minutes at 100° C. Kidder (1941) asserts that this substance in maximum concentration, produced by *Tetrahymena geleii*,[10] disintegrates completely in 5 minutes at 100° C.; and Marbarger (1943) asserts that the growth-substance produced by *Colpidium striatum* disintegrates only slightly, if at all, in 2 hours at 100° C. The following experiments concern this problem.

TABLE 6

THE EFFECT OF OXYGEN ON GROWTH-SUBSTANCE PRODUCED BY *Chilomonas paramecium*. AC-AM, ACETATE AMMONIUM SOLUTION; FRESH, AC-AM SOLUTION IN WHICH NO CHILOMONADS HAD BEEN; OLD UNTREATED, AC-AM SOLUTION IN WHICH A CULTURE OF CHILOMONADS HAD DEVELOPED AND DIED OUT; OLD O_2-TREATED, SAME EXCEPT THAT PURE OXYGEN GAS HAD PASSED THROUGH IT FOR 5 HOURS; OLD N_2-TREATED, SAME EXCEPT THAT PURE NITROGEN GAS HAD PASSED THROUGH IT FOR 5 HOURS. SIXTEEN ISOLATED CHILOMONADS WERE USED IN EACH TEST

Ac-am Culture Fluid	Average Time in Hours Required for First Generation in:	
	Expt. A	Expt. B
Fresh	6.45±0.21	6.18±0.18
Old untreated	10.15±0.43	10.72±0.37
Old N_2-treated	9.80±0.24	10.60±0.39
Old O_2-treated	9.25±0.22	9.00±0.23

Fluid in which a culture of chilomonads had died out was obtained as in the preceding experiments. This fluid consequently contained growth-substance in maximum concentration. An equal amount of it was put into each of 6 small testtubes and the same amount of fresh ac-am solution in each of 2. Then all were suspended in constant-temperature water-baths; 1 of the latter at 24° C. and the other at 100° C.; and the former 6, respectively, at 24°, 37°, 45°, 65°, 85°, and 100° C. After 15 minutes the rate of growth of *Chilomonas* in solution from each of the tubes was measured as described above. This experiment was repeated 3 times, except that the solutions were subjected longer to the different temperatures and that, in 1, only 2, instead of 6, testtubes were used for old culture fluid and these were subjected to 24° and 0°, respectively. The results obtained are recorded in Table 7.

Table 7 shows very clearly that, if the inhibition of growth in old culture fluid is due to the presence of an excessive

[10] Pace (1944) asserts that Marbarger and Kidder studied the same species, and Dewey (1944) supports this assertion. The organisms studied by these two investigators doubtless belong to a small group in the family Frontoniidae, but the identification of the members of this group is so difficult and their classification so confused that their precise phylogenetic interrelationship is, at best, far from certain.

Marbarger says: "The organisms investigated correspond with the description of those designated *Colpidium striatum* by Stokes." The description of Stokes (1888) is, however, inadequate for precise identification, and the cultures from which Marbarger obtained his organisms have unfortunately died out.

Kidder maintains that the organisms he studied agree with Furgason's description of the proposed new genus *Tetrahymena* (1940). This description is, however, so nearly in accord with that of the genus *Leucophrys* Ehrenberg (1838) that it is difficult to see how the formation of a new genus can be justified. There are, moreover, serious objections to the form of the name of the new genus. Furgason says the name *Tetrahymena* (four membranes) was selected owing to the presence of four membranes in the region of the mouth. The plural of *hymen* (membrane) is, however, *hymenes*, not *hymena;* but, according to the International Rules of Zoölogical Nomenclature, generic names must be nominative singular, not plural. Therefore, if the proposed new genus is accepted, its name should be *Tetrahymen*, not *Tetrahymena*.

amount of growth-substance, as Mast and Pace (1938) maintain, the rate of disintegration varies directly with the temperature and the amount disintegrated directly with the time of exposure. The results obtained with old culture fluid exposed for 5 hours at 100° appear to contradict these conclusions, for the generation-time obtained under these conditions appears to be higher, instead of lower, than that obtained with 5 hours' subjected to 85° for 5 hours. This seems to indicate that exposure to 100° for 1 hour or to 85° for 5 hours caused disintegration of all the growth-substance in the old culture fluid. This is, however, not certain; for, according to the contention of Mast and Pace, as the concentration of growth-substance decreases from maximum, the rate of growth increases to a maximum which is higher than that in fresh ac-am solution and then decreases, i.e., the rate of growth in any suboptimum concentration of growth-substance is the same as it is for some superoptimum concentration, so that if the rate of growth in old culture fluid has been increased, owing to destruction of growth-substance, from zero to that which obtains in fresh ac-am solution, the concentration of this substance may be zero or it may be superoptimum. To be certain that it is the former, it must be known that the rate increased beyond that which obtains in fresh ac-am solution and then decreased (Fig. 2).

TABLE 7

THE EFFECT OF TEMPERATURE ON GROWTH-SUBSTANCE PRODUCED BY *Chilomonas paramecium*. AC-AM, ACETATE-AMMONIUM SOLUTION; FRESH, AC-AM SOLUTION IN WHICH NO CHILOMONADS HAD BEEN; OLD, AC-AM SOLUTION IN WHICH A CULTURE OF CHILOMONADS HAD DEVELOPED AND DIED OUT. IN A AND B EACH LENGTH OF TIME GIVEN IS THE AVERAGE FOR 32 AND IN C AND D FOR 48 CHILOMONADS

AC-AM CULTURE FLUID	AVERAGE TIME IN HOURS REQUIRED AT 24° C. FOR FIRST GENERATION IN:			
	Expt. A (Exposed 15 Min.)	Expt. B (Exposed 60 Min.)	Expt. C (Exposed 5 Hr.)	Expt. D (Exposed 21 Days)
Fresh at 24° C.	6.45±0.21	6.14±0.13	7.00±0.09	6.43±0.17
Fresh at 100° C.	6.32±0.18	6.22±0.17	7.15±0.14	.....
Old at 0° C.	.....	.....	.....	9.15±0.24
Old at 24° C.	10.25±0.36	10.92±0.20	9.55±0.23	7.37±0.20
Old at 37° C.	11.00±0.32	10.54±0.21	8.54±0.18	.....
Old at 45° C.	10.08±0.31	9.16±0.17	8.29±0.20	.....
Old at 65° C.	9.82±0.29	8.54±0.24	7.38±0.18	.....
Old at 85° C.	8.90±0.17	6.51±0.17	6.78±0.11	.....
Old at 100° C.	8.72±0.13	6.32±0.15	6.97±0.12	.....

exposure at 85° and that obtained with 60 minutes' exposure at 100°. This is, however, in all probability due to disintegration of so much growth-substance that what remained induced acceleration instead of retardation in the rate of growth. If this is true, the results obtained with 5 hours' exposure at 100° are in harmony with the conclusions presented.

Table 7 shows that in old culture fluid which had been subjected to 100° for 1 hour, the generation-time at 24° was nearly, if not quite, the same as it was in fresh culture fluid and that this is also true for old culture fluid which had been

Neither the results presented by Mast and Pace (1938) nor those presented by

Kidder (1941) nor those presented by Marbarger (1943) show that this obtained. Their conclusion regarding the extent of disintegration of growth-substance are consequently equivocal.

Table 7 shows also that in the old culture fluid which had been exposed at 24° the conclusion reached in a preceding section, namely, that the growth-substance produced by *Chilomonas* disintegrates at room temperature.

Table 7 also shows that the generation-time in old culture fluid subjected to 0° was reduced approximately from

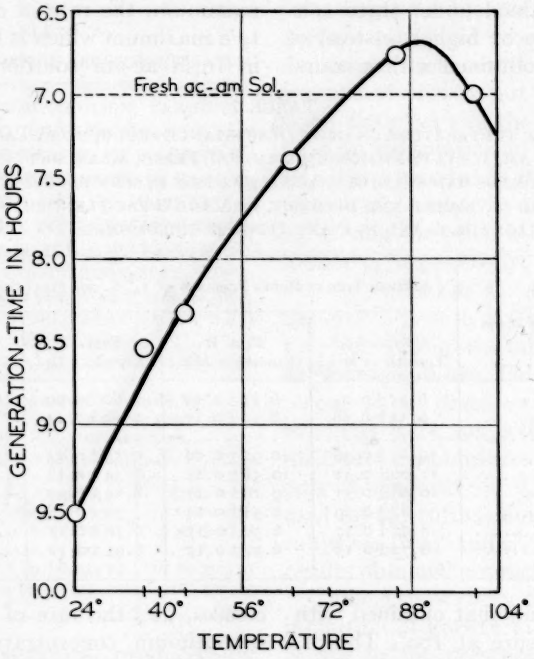

FIG. 2.—The effect of heat on growth-substance produced by *Chilomonas* as indicated by the rate of growth at 24° C. in old culture fluid which had been subjected for 5 hours to different temperatures. Note that, as the temperature increased, the generation-time decreased to a minimum (which was lower than that in fresh ac-am solution) and then increased.

for 15 minutes, the generation-time of chilomonads at 24° was 10.25 hours but that in the old culture fluid which had been exposed at this temperature for 3 weeks, it was only 7.37 hours. That is, it indicates that, as the time old culture fluid is kept at room temperature increases, the retarding effect of the growth-substance in it decreases and the rate of growth increases. This supports 10.25 hours to 9.15 hours in 3 weeks. This indicates that chilomonad growth-substance disintegrates at 0° but very slowly.

Figure 2 indicates that in old culture fluid which has been subjected for 5 hours to different temperatures, respectively, the rate of growth of chilomonads at 24° increases (with increase in the temperature to which the fluid had been exposed)

to a maximum which is higher than the rate of growth in fresh culture solution and then decreases. If this is true, it strongly supports the contention of Mast and Pace (1938) that *Chilomonas* produces a substance which has a retarding effect on growth if it is relatively high in concentration and an accelerating effect if it is relatively low.

SUMMARY

1. The results obtained support the contention of Mast and Pace (1938) that *Chilomonas paramecium* produces a substance which in low concentration accelerates, and in high concentration retards and inhibits, growth and reproduction.

2. This substance, called "growth-substance," readily passes through a Cellophane membrane, containing pores approximately 6 mμ in diameter. The molecules of this substance are probably smaller than those of sucrose.

3. The growth-substance gradually disintegrates if culture fluid containing it is exposed to air at room temperature.

4. Increase in temperature, evaporation of the water in the culture, or exposure of the dry residue to pure oxygen or air increases the rate of disingegration.

5. The disintegration of the growth-substance is probably due to oxidation.

6. The rate of disintegration of growth-substance varies directly with the temperature. At 0° C. it is very low but still measurable. At 100° C. probably all, in culture fluid containing a maximum concentration, disintegrates in 5 hours and possibly in 1 hour.

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PHYSIOLOGY OF THE CILIATE COLPIDIUM COLPODA. III. THE POSSIBLE USE OF THE DIVISION RATE OF COLPIDIUM COLPODA FOR IDENTIFICATION OF INTESTINAL BACTERIA[1]

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A FAMILIAR definition of the species concept states that a species is a homogeneous population that is genetically distinct and reproductively isolated. The precise application of this definition to difficult taxonomic problems concerning the higher sexual organisms is tedious but often valuable. When any attempt is made to deal taxonomically with such organisms as bacteria, which are asexual and apparently lack a discrete nucleus, even the species concept itself becomes obscure (Henrici, 1939).

Although the difficulties attending bacterial taxonomy are great, yet it is possible to correlate information gained from using standard methods for the classification of bacteria with certain evidence from allied fields. In an earlier reconnaissance of bacterial-protozoan relationships by Burbanck (1942*a*), many suspensions of different species of bacteria were used as food for the ciliate *Colpidium colpoda*. In spite of the fact that only eight lines of colpidia were grown on suspensions of each of thirty bacteria, there was a definite and often statistically significant tendency for colpidia to have different division rates on suspensions of bacteria belonging to different genera and even different species of the same genus. With this in mind, a new problem can now be attacked: Can the division rate of this protozoan be useful as a means of separating species of bacteria? In making such an attempt, one must remember:

> Whether a given character shall be considered of taxonomic value depends upon whether it varies in a perfectly continuous manner or shows a tendency to discontinuous variation, and whether it is correlated with changes in other characters, or varies independently of other characters. These things may be learned by the use of *statistical methods* [Henrici, 1939].

In the earlier work by Burbanck (1942*a*) it was found that colpidia grown on suspensions of two taxonomically distinct genera, *Pseudomonas fluorescens* and *Aerobacter aerogenes*, showed very striking differences in their division rates. The mean division rates, when tested statistically by the use of the methods developed by "Student" (1925) and R. A. Fisher (1925), showed good evidence of difference, i.e., there was a discontinuous variation. Thus it will be seen that the division rate of *Colpidium* may be used as a test for the separation of at least certain genera of bacteria and that this evidence is related to Woodruff's (1925) statement that fission rate is the "most subtle index of metabolism."

With the use of this ability of *Colpidium* to divide at different rates when

[1] We are indebted to the American Philosophical Society for the support of this work by a grant-in-aid.

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grown on suspensions of different bacteria, the indications arising from the preliminary results gained from the earlier work and the more precise information based on the two bacteria, *Pseudomonas* and *Aerobacter*, may be put to the test to discover whether the "protozoan method" may serve as another check to supplement the already well-established and often effective methods of determinative bacteriology.

MATERIALS AND METHODS[3]

Since this was to be the test of a new method, experimental bacteria were purposely chosen which were pathogenic for man and very similar in their antigenic structure and fermentation reactions. Both the control bacterium and the experimentals were taken from one of the six families formerly studied, which contained species of bacteria that uniformly supported the highest division rates of *C. colpoda*. This family is the Enterobacteriaceae. Although ten species from this family had been studied before (Burbanck, 1942*a*), the present work was planned to be of a more intensive than extensive nature; consequently, the following bacteria were chosen: *Escherichia coli* (No. 26), *Eberthella typhosa* (No. 6539), *Salmonella paratyphi A* (No. 6374), and *Shigella dysenteriae* (No. 731). The last-mentioned was to be tested for the first time in experiments of this sort. All these cultures were procured from the American Type Culture Collection.

Among these intestinal bacteria, *E. coli* was chosen as the most appropriate food for the control colpidia, because in earlier work (Burbanck, 1942*a*) it was found that colpidia grown on suspensions of *E. coli* had a more constant division rate than those grown on either *Eb. typhosa* or *Sal. paratyphi A*. Also, in spite of the close morphological and physiological resemblance to the three experimental bacteria, *E. coli* is set apart from them, since it is a normal harmless commensal of the human intestinal tract, while the others are all virulent parasites.

For this series of experiments, colpidia were taken from a hay-tea culture which had been subcultured monthly since the original strain was obtained in 1939. These colpidia were washed according to Parpart's method (1928) and grown on suspensions of living *P. fluorescens*[4] which had a viable count of 100,000,000 per milliliter. At this point a departure was made from the procedure of the earlier work (Burbanck, 1942*a*). Instead of keeping the stock cultures of animals on suspensions of living *Pseudomonas*, stocks of *Colpidium* were maintained at a reduced division rate on a nonliving medium (Burbanck, 1942*b*). Colpidia taken from suspensions of living *Pseudomonas* were washed in the same manner as described above and grown in isolation cultures on suspensions of killed *P. fluorescens* (10 minutes at 60° C.) plus yeast (prepared as described by Burbanck [1942*b*]), and all the protozoans used in subsequent tests were from the strain held on this nonliving medium.

The techniques used in handling the protozoa and the bacteria during the present experiments were essentially the same as those described earlier, except that the sterile watch glasses used for the isolation cultures were now kept in individual Petri dishes rather than in one large crystallizing dish. Each isolation culture was continued for 7 days with

[3] We wish to thank Professor R. Neal and Mr. H. Bender for the loan of apparatus from their respective departments of chemistry and geology at Drury College.

[4] Obtained from the department of bacteriology of the University of Chicago.

daily subcultures of one animal per line. The animals were placed in 0.2 ml. of bacterial suspension, which was standardized at approximately 100,000,000 viable bacteria per milliliter. Total counts of *C. colpoda* were made every 24 hours. Each experimental unit consisted of four lines of *C. colpoda* grown on suspensions of the control and three experimental bacteria. Twelve separate runs, using the isolation technique, were performed, totaling forty-eight separate lines. At the end of each 7-day period in the above runs and at the end of each 3-day period in the next series of experiments, the hydrogen-ion concentration was taken by the use of a Hellige comparator.

In addition to the isolation-culture experiments, twelve separate lines of *C. colpoda* were grown on the same four bacteria in 3-day mass cultures. A mass-culture experiment consisted of one *C. colpoda* in 1 milliliter of bacterial suspension of approximately 100,000,000 viable bacteria per milliliter. The single animals were grown as in the isolation-culture experiments in sterile watch glasses inclosed in Petri dishes. At the end of 3 days, all the progeny were counted.

The data from these experiments (both isolation and mass culture) were analyzed statistically according to the methods of "Student" (1925) and R. A. Fisher (1925).

RESULTS AND DISCUSSION

Tables 1 and 2 show the results of the forty-eight 7-day isolation cultures of *C. colpoda* grown on suspensions of the control bacterium, *E. coli*, and on the three experimental bacteria, *Shig. dysenteriae*, *Eb. typhosa*, and *Sal. paratyphi A*. The division rates of *C. colpoda* grown on the experimental bacteria are listed in chronological order, with two exceptions. The four lines of *Shig. dysenteriae* in experimental unit 3 showed contamination and were repeated later, and the figures inserted in the table are from the replacement. Experimental units 9 and 10 (again *Shig. dysenteriae*) were repeated because a recheck on the bacterial count revealed that the concentration was below the standard of 100,000,000 per milliliter. In Table 2 the results of the twelve tests, of four lines each, have been combined, and the average daily division rate of *C. colpoda* grown on one species of bacterium has been compared with the average daily division rate on each other bacterial species. The division rates are significantly different, as indicated by the recurrence of $P=0.0000$; P-value of less than 0.05 is often considered to indicate a real difference statistically; and the smaller the value of P, the higher is the probability of there being a real difference in the compared values.

Tables 3 and 4 show the results of the twelve mass-culture experiments with the same organisms. Each division rate listed in these two tables represents the average number of divisions in 3 days, not in 1 day, since total counts were made of mass cultures at the end of 3 days rather than daily as in the isolation-culture experiments. The data from Table 3 have been summarized and analyzed in Table 4. Again the division rates vary significantly, but the order from highest to lowest is different from that in the isolation-culture experiments. These data on mass cultures should be considered as a preliminary study, and further work needs to be done to test the results recorded in Table 4.

Inspection of Table 1 reveals that the division rate of *C. colpoda* decreased as the experiments proceeded. It is difficult to explain this decrease, which occurred to about the same degree for all four

TABLE 1

DIVISION RATES OF *C. colpoda* GROWN IN ISOLATION CULTURES ON FOUR SPECIES OF BACTERIA

Experimental Units	Line	Average Daily Division Rate for 7-Day Period			
		Control	Experimentals		
		E. coli	*Shig. dysenteriae*	*Eb. typhosa*	*Sal. paratyphi A*
1	1	2.2151	3.2750	2.4296	2.3693
	2	1.4011	2.8507	2.4771	2.2825
	3	2.5989	3.1824	2.2328	2.1399
	4	2.8348	3.2370	2.2967	2.2825
2	5	2.2787	2.9734	2.9661	1.8062
	6	2.2449	2.7253	2.6702	2.0899
	7	2.5633	2.5307	3.0920	1.6550
	8	2.5714	2.9777	2.7138	1.8814
3	9	2.3735	2.8307*	2.4286	2.0243
	10	2.1888	2.7063*	2.5714	1.8438
	11	2.1422	2.5866*	2.4286	1.9375
	12	2.5306	2.7746*	2.6591	2.1153
4	13	2.1153	2.2993	2.5121	2.1020
	14	2.1296	2.3560	2.4470	2.0327
	15	2.2264	1.4011	2.5867	1.3693
	16	2.1162	2.2131	2.4571	1.3693
5	17	1.5364	3.1264	2.6238	2.3227
	18	1.8221	3.1990	2.2307	1.9734
	19	2.4253	3.2562	2.4846	1.9725
	20	1.7845	3.1539	2.5952	2.0485
6	21	1.7386	2.8871	2.0836	1.5714
	22	1.6550	2.8030	1.5581	1.6174
	23	1.0243	2.8164	1.6550	2.1153
	24	1.5364	2.9412	1.8539	1.8438
7	25	1.5957	2.4470	2.0243	1.8464
	26	1.7978	2.1888	2.0243	1.6517
	27	1.8221	2.4470	1.9132	1.8681
	28	1.9592	2.1428	1.7978	1.6417
8	29	1.7385	2.4846	1.9031	1.6417
	30	2.0836	1.5957	1.8438	1.7385
	31	1.9734	2.0000	2.3100	1.1078
	32	1.7143	2.1714	1.9650	1.4285
9	33	1.8438	1.6550†	2.3978	1.8571
	34	1.9407	1.6550†	2.1281	1.8814
	35	1.5956	1.5957†	2.1162	1.6417
	36	1.6550	1.5089†	1.9132	1.9132
10	37	2.1396	1.7469†	2.1989	1.6550
	38	1.9650	1.7010†	2.3285	1.5957
	39	1.5364	1.5957†	1.7929	1.5364
	40	1.5957	1.7010†	2.3418	1.4528
11	41	1.5956	1.8571	1.4286	1.3100
	42	1.4528	1.8296	1.3100	1.6792
	43	1.8571	2.0243	1.3100	1.4286
	44	1.6868	1.8364	1.7143	1.6792
12	45	1.9867	2.2215	1.6174	1.3693
	46	1.8571	2.0836	1.3935	1.1429
	47	1.8221	2.1989	1.6550	1.4528
	48	1.8814	2.0243	2.1121	1.3693

* The original runs showed contamination, and the experiment was repeated in conjunction with Expt. 5.

† The original runs did not contain the standard bacterial concentrations, and the experiments were repeated in conjunction with Expt. 12.

TABLE 2

STATISTICAL COMPARISON OF AVERAGE DAILY DIVISION RATES OF FORTY-EIGHT LINES OF *C. colpoda* GROWN IN ISOLATION CULTURES FOR 7-DAY PERIODS ON FOUR SPECIES OF BACTERIA

	Av. Daily Div. Rate	Control	Experimentals		
		E. coli	*Shig. dysenteriae*	*Eb. typhosa*	*Sal. paratyphi A*
		1.9406	2.3712	2.1582	1.7713
E. coli	1.9406	.....	$P=0.0000$	$P=0.0000$	$P=0.0000$
Shig. dysenteriae	2.3712	.....	.....	$P=0.0000$	$P=0.0000$
Eb. typhosa	2.1582	.....	.....	.....	$P=0.0000$
Sal. paratyphi A	1.7713	.....	.....	.....	.....

TABLE 3

DIVISION RATES OF *C. colpoda* GROWN IN MASS CULTURES ON FOUR SPECIES OF BACTERIA

Experimental Units	Average Division Rate for 3-Day Period			
	Control	Experimentals		
	E. coli	*Eb. typhosa*	*Shig. dysenteriae*	*Sal. paratyphi A*
1	5.3927	5.7555	7.6375	6.0449
2	4.3219	6.0007	7.2485	6.0007
3	6.5399	7.9375	6.5857	4.3923
4	4.7010	8.2535	7.0120	5.0449
5	3.3219	7.0007	6.7950	5.5551
6	2.5850	6.6007	6.2674	5.8585
7	3.3219	7.8834	6.1505	5.4269
8	3.8074	7.1504	5.8834	5.6730
9	4.4594	8.1554	6.3405	4.3923
10	4.5853	6.3405	6.2296	5.3581
11	4.3923	7.2485	5.8834	5.4924
12	5.2100	7.0087	5.9312	4.9548

TABLE 4

STATISTICAL COMPARISON OF AVERAGE DIVISION RATES OF *C. colpoda* GROWN IN MASS CULTURES ON FOUR SPECIES OF BACTERIA FOR TWELVE 3-DAY PERIODS

	Av. 3-Day Div. Rate	Control	Experimentals		
		E. coli	*Eb. typhosa*	*Shig dysenteriae*	*Sal. paratyphi A*
		4.3866	7.1188	6.4971	5.3495
E. coli	4.3866	.....	$P=0.0000$	$P=0.0000$	$P=0.0000$
Eb. typhosa	7.1188	.....	.....	$P=0.0001$	$P=0.0000$
Shig. dysenteriae	6.4971	.....	.....	.....	$P=0.0000$
Sal. paratyphi A	5.3495	.....	.....	.....	.....

species of bacteria. Experimental conditions were standardized in every way possible. Gram stains were made at the end of each experiment to make sure that the cultures were free from contamination. The standard concentration of the bacterial suspensions was maintained. During the 7-day experimental periods the Petri dishes containing the experimental animals were kept in an incubator at a temperature of $25.5 \pm 0.5°$ C. The pH was taken at the end of each experiment; and for thirty of the forty-eight experiments the variation was not great for an unbuffered system but ranged from 7.1 to 7.3. A low reading of 6.7 was recorded only once, and the high of 7.6 occurred four times. These high readings were for the last two experiments with *Shig. dysenteriae* and *Eb. typhosa*. Readings below 7.0 occurred during the first five experiments, and readings above 7.3 occurred during the last five experiments, with one exception. Thus there would seem to be some correlation between lowered division rates and increase in alkalinity. The increase in alkalinity may have resulted from an increase in the density of the bacterial suspensions. The suspensions appeared to be more dense during the latter half of the experiments, but there were no means available to make an accurate analysis of them, and the *viable* count was maintained at the standard for these experiments. The variation in pH cannot be considered as the cause but only as a possible influence upon the rate of division of *C. colpoda*. It is possible that the decrease in division rate was caused by some other physiological change which affected all the animals.

The results of these experiments bear out the earlier suggestion (Burbanck, 1942*a*) that different species of bacteria cause significant differences in the division rate of *C. colpoda*. The bacteria used were four species which may live in the human intestines. Three of them are considered pathogenic or abnormal inhabitants, and one, *E. coli*, is normally found in this habitat. Although the ability of the bacteria to support *C. colpoda* varied in order, depending on whether the experimental method used was isolation or mass culture, yet, within a given method of culture, in every case there was a *significant* difference between the rate of division of the protozoan when grown on any two species of these bacteria. In isolation cultures, *Shig. dysenteriae* was most effective, followed in order by *Eb. typhosa*, *E. coli*, and *Sal. paratyphi A*. In mass cultures the order of effectiveness became *Eb. typhosa*, *Shig. dysenteriae*, *Sal. paratyphi A*, and *E. coli*. This variation in order may result from some influence based on the altered ratios of volume and exposed surface or from the longer time interval for the mass cultures; or there is a somewhat remote possibility that it is an artifact related to the smaller number of replications of the mass-culture experiments. The fact remains that the isolation-culture method, and probably the mass-culture method also, could be used to separate the species of bacteria, should such a check be needed on their isolation and identification. Since the bacteria used cause typhoid fever, food poisoning, and dysentery and are therefore important to man and since their identification is sometimes difficult by usual methods, the differential reaction to them as food by *C. colpoda*, despite the time and labor involved, may prove of value to determinative bacteriology. The use of a nonliving medium for stock cultures of *C. colpoda* simplifies and shortens the steps necessary to maintain a sterile and controlled experiment which is so important

in work of this nature. It is possible that the physiological reactions of *C. colpoda* to different bacteria as food might prove useful, at least as a check on the identification of other bacteria in addition to those reported upon in this paper.

Finally, the "protozoan method" may be compared to a very helpful technique used in the taxonomy of higher plants and animals, viz., the study of chromosome numbers and configurations. The counting and study of chromosome morphology and arrangement is a difficult technique, demanding skilled workers; but it has added immeasurably to our understanding of confused species, which have many intergrading characteristics. Chromosome study will never replace characteristics used in less exacting identifications; but, for the careful taxonomist, the structure, arrangement, and number of chromosomes in any species will become more and more important as a fundamental characteristic. So it is with the use of the reactions of protozoans in the identification of certain bacteria. Although the isolation-technique method is a very accurate, but somewhat tedious, method for obtaining the reaction of a protozoan to a specific bacterium, it may be worth the extra effort for more careful taxonomy. The 3-day mass-culture method, on the other hand, with further testing may prove to be equally good; or perhaps adequate tests using protozoa may be run in 24 hours if a sufficiently large number of separate parallel experiments are set up at one time.

SUMMARY

1. Four species of intestinal bacteria caused significant differences in the division rate of *C. colpoda* in isolation cultures.

2. The same four bacterial species caused significant differences in the division rate of *C. colpoda* in this preliminary study with the small mass-culture technique.

3. The use of a nonliving medium for stock-culturing *C. colpoda* simplifies the experimental procedure.

4. It is suggested that the division rate of *C. colpoda* could be employed at least as a means of checking on the separation of species of bacteria in careful taxonomic studies and in some phases of medical diagnosis.

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HOMOTYPIC AND HETEROTYPIC CONDITIONING IN RELATION TO SURVIVAL AND GROWTH OF CERTAIN FISHES[1]

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ORGANISMS often react on their common environment and produce a changed medium that differs decidedly from one that has never been occupied by living things. The present paper is limited to the report of a recent set of laboratory studies concerning certain effects of fish-conditioned water upon other fishes. Like aquatic animals in general, fish remove dissolved oxygen and take up food from the surrounding water. They give off wastes, including carbon dioxide, urea, and more or less solid feces. They often alter the inorganic salt content of water, especially in small laboratory aquaria. For example, fish introduce NH_3, NO_2, NO_3, and organic nitrogen into some waters and increase the total and the fixed residues on evaporation (Allee, Bowen, Welty, and Oesting, 1934).

Fish secrete mucus that may adhere to the body surfaces or may become free in the water or be deposited on the walls of the container, or on stones and plants in nature. If well fed, many kinds of fishes regurgitate food particles and, during the breeding season, the majority of them shed eggs and sperm. The mucus, regurgitated food, sexual products, and feces act as adsorbing surfaces for various ions, including toxic ions of the heavy metals, and effectively remove them from the active environment. They alter the bacterial flora of water in which they live. Some species profoundly affect the more superficial layers of the underlying substrate. These are the major reactions of fish upon their fresh-water environments.

Homotypic conditioning is produced by animals of the same species, and heterotypic conditioning by animals of other species than that on which the effects are being tested. The latter category includes conditioning by plants and by animals widely separated in the taxonomic series from those under assay, as well as by more closely related species. The long-continued investigation in this laboratory of the relations between conditioned water and the growth of fishes has dealt mainly with intraspecific effects. Homotypically conditioned water, in the early stages of conditioning, often promotes more rapid growth than does a comparable, unconditioned medium (see citations, p. 257).

A synthetic pond water was developed early in the course of these studies in order to secure a reproducible medium that had never been inhabited. It is made by adding the following analyzed-grade chemicals in the given amounts per liter of water: 100 mg. $CaCl_2 \cdot 4H_2O$; 50 mg. each of $NaNO_3$, $MgSO_4 \cdot 7H_2O$, and K_2SO4. In addition to demonstrating accelerated growth from homotypic conditioning, Allee, Oesting, and Hoskins (1936) and Allee, Finkel, and Hoskins (1940) found that goldfish grew better in this synthetic pond water when

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an extract from the surface of other goldfish was added in concentrations as dilute as 1 part in 800,000 of water than they did in untreated synthetic pond water.

An analysis of the several factors that may be involved in the effect of homotypically conditioned water on growth of fresh-water fishes has been partially successful (p. 254). The fixation of toxic materials has been clearly demonstrated (Allee and Bowen, 1932), and food regurgitation also plays a considerable part (Allee, *et al.*, 1936; Livengood, 1937). There is a possibility that certain compounds present in conditioned water have a more specific growth-promoting effect. No such substance has been isolated, although the experiments with skin extracts seem to suggest its presence.

The general experience of aquarists indicates that heterotypically conditioned water is often an improved medium for fish culture when the conditioning organisms are not closely related to the test animals. Shaw (1932) studied some phases of interphylum conditioning; she found that the growth of the fish *Platypoecilus*, and the regeneration of the tails of certain salamander larvae (*Ambystoma*) are accelerated in water in which fresh-water mussels have lived, as compared with unconditioned well water, even when the mussel-conditioned medium was passed through coarse filter paper to remove masses of mucus. The tadpole tails regenerated more rapidly in the conditioned water than in well-water controls. Shaw found it easier to demonstrate acceleration of growth in mussel- than in self-conditioned medium.

Less experimental attention has been given to the results obtained by the use of conditioning animals that are more closely related taxonomically to the species under assay. There is a preliminary report that the rate of growth of goldfish is increased by water in which mud minnows have lived, as compared with that shown in untreated synthetic pond water (Allee, Garner, and Guhl, 1942).

The present experiments were planned to investigate the latter aspect of interspecific conditioning among fishes. Since much of the previous work in this laboratory has been concerned with the growth of goldfish, they were again used as assay animals both in homotypically and in heterotypically conditioned water. Similar and often simultaneous experiments were made with some other common fresh-water fish. The experiments were carried on intermittently from October, 1941, into early 1946. During parts of this period many deaths occurred among the experimental and control fish as a result of some toxic material in the water. This complication causes some of our data to deal with the effects of conditioning on survival and decidedly negative growth (decrease rather than increase in weight or length). It must be emphasized that the factors operating under these conditions may differ in relative importance from those found to be important in the earlier experiments in this series.

ANIMALS AND THEIR TREATMENT

The goldfish (*Carassius auratus*) used were of the single-tailed variety. They were purchased in lots of 100 or more from a single hatchery. Bullheads (*Ameiurus melas*), mud minnows (*Umbra limi*), and common shiners (*Notropis cornutus*) were obtained by seining in suitable habitats relatively near Chicago. Within 48 hours of being introduced into the laboratory, all fish were treated with a dilute solution of potassium permanganate to remove some of the parasites

that were of rather frequent occurrence. The goldfish were usually treated, in addition, with 0.4 per cent acetic acid or with a 1 per cent solution of sodium chloride. Subsequently, the fish were kept either in laboratory aquaria or in out-of-door cement tanks, heated in winter. Care was taken that fish used in an experiment had been in the laboratory for at least 2 weeks. Growth of the same individuals was assayed only once, and the fish used to condition the water had rest periods of a month for each month they acted as conditioners.

The synthetic pond water, which we used as an experimental medium, must be produced in large quantities. This requires a steady supply of distilled water, which is produced in a steam-heated still that has its exposed copper surfaces covered with aluminum. The lining was replaced shortly before these experiments were begun and again in August, 1945.

Since Chicago's water is hard and heavily chlorinated, the supply line to the still leads first through a "Refinite" water-softener and then an activated charcoal filter. The distilled water usually had a conductivity of less than 4×10^{-6} reciprocal ohms; often it reached 2×10^{-6}. Despite much care, the distilled water varied somewhat from time to time, partly as a result of a gradual wearing-away of the aluminum lining with continued use and repeated cleaning.

Some toxic factor, which we have not been able to identify with precision, has been operating during a part of the experiments included in the present report. Evidence concerning the toxic substance will be presented on page 247.

Goldfish, shiners, mud minnows, and bullheads served as assay animals at various times; only one species was assayed in any one experiment, although two interlocking experiments were often conducted simultaneously. In a typical test, equal numbers of fish were isolated in earthenware jars of 1-gallon capacity, containing 1 liter of one of the following media: (1) homotypically conditioned water; (2) heterotypically conditioned water; or (3) unconditioned water. Survival and growth in the three kinds of water were compared at the end of assay periods that lasted for 20 days.

In Experiments PF2–8, fingerbowls containing 200 cc. of water were substituted for the gallon jars, since small bullheads were being assayed. The results obtained with this procedure were similar to the rest. Three types of heterotypic conditioning were employed in the experiments with goldfish as assay animals: shiner-conditioned, mud minnow–conditioned, and bullhead-conditioned water. For the other fishes, heterotypic conditioning was accomplished by using goldfish-conditioned water.

The experimental jars were placed in trays containing $1\frac{1}{2}$ inches of running water, in an attempt to keep temperature changes to a minimum in each assay period. The paired jars for experimental and control fish were so arranged as to compensate for differential lighting and other differences as much as possible. Although the temperature of the water tray varied from a winter low of about 10° to a summer high of approximately 24° C., the temperature variation in any one experiment was negligible (for Expt. PF13, for instance, C.V. = 8.5).

As in previous work, the assay fish were transferred to fresh water of the proper type every 24 hours; they were fed for 2–3 hours previous to each change. The empty jars were washed out and were wiped with soft towels. A small quantity of chemicals may have

been introduced during the wiping, since the towels were usually not re-washed after their return from the laundry.

The food for the bullheads and the mud minnows under assay consisted of Enchytraeidae; that for the rest of the fish was rolled oats, in 1941 and 1942, or a mixture of cooked corn meal and soybean meal. All fish were fed ground, raw liver at somewhat irregular intervals, but not during the assay period. Conditioning goldfish and bullheads were fed the mixture of corn and soy meal, except that in Experiment PF18 bullhead conditioners were fed liver; this experiment dealt primarily with growth in the medium, based on twice-distilled water.

All assay fish, except the bullheads in Series B 1–15, and the shiners in B 17, were photographed at the beginning and, except in some of the survival experiments, at the end of the 20-day assay period, in order to measure changes in length. The fish in the B series mentioned above were weighed to obtain growth measurements. Some of the assay goldfish were weighed, in addition to being photographed. The procedure for the photographic method of making growth measurements has been described in some detail (Allee *et al.*, 1934). For weighing, the fish were wrapped in a small piece of moist cloth, which was then placed in a clean, covered soapdish. This was weighed and the fish then removed. Care was taken that the fish received a minimum of handling. The soapdish and the cloth were then re-weighed. The standard error of the mean in weighing was $\pm$ 0.52 per cent for the bullheads and $\pm$ 0.15 per cent for the goldfish. This is a marked reduction from the variation reported in 1934 (*ibid.*, p. 187).

Assay goldfish ranged from 3.5 to 5 cm. in length; mud minnows, from 4.5 to 7 cm.; shiners, from 4 to 5 cm.; and bullheads, from 2.5 to 4 cm., except in one experiment, in which they were approximately 8 cm. long.

The procedures involved in conditioning the water follow closely those used previously (Allee, Finkel, and Hoskins, 1940). To review them briefly: conditioning fish are placed in large earthenware jars containing 5 liters of water. They remain in this water, which is steadily aerated, for 22 hours. They are then removed, fed in other earthenware jars, rinsed, and put into fresh, uncontaminated water. The conditioned water is now filtered through a phosphobronze sieve with 325 meshes to the inch, diluted to give a conditioning coefficient of 25 (p. 251), and distributed to the experimental jars. The same amount of unconditioned water, treated similarly except that it contains no conditioning fish, forms the medium for the accompanying controls.

Two experiments were performed with skin extracts, using an alkaline goldfish extract similar to that described by Allee *et al.* (1940). An extract from bullheads was prepared by the same method. One or the other of these extracts was placed in synthetic pond water. Sodium hydroxide was added to the control jars in concentrations comparable to those of the extracts to prepare a control medium with the same alkalinity found in the diluted extracts. The experimental procedure in regard to measuring the fish, feeding, changing of the water, etc., was otherwise the same as for the conditioning experiments.

The measurements and survival values obtained were analyzed statistically by the *t*-test, using the distribution of "Student." *P*-values of 0.05 were taken as the upper margin of statistical significance.

EXPERIMENTAL RESULTS

PROTECTION FROM TOXICITY

The general trend of the experimental results—using conditioned water made from single-distilled water, under the conditions described—has been for longer survival in conditioned than in unconditioned water. Homotypic and heterotypic conditioning produced similar results. No series of experiments reverses this trend, although individual tests did not always agree with it in detail.

Bullheads were used as assay animals in the survival series, in which most data were accumulated. The results are summarized in Table 1. When compared with the total time that the fish could have lived if all had survived to the end of the given experiment, the bullheads lived, on the average, nearly 14 per cent longer in homotypically conditioned water than in untreated, control water. The difference has good statistical significance, with a *P*-value of 0.01 when computed by paired experiments and of 0.0000 by paired fish. The comparison on the same basis between the bullheads in goldfish-conditioned water and their controls shows a much smaller mean difference; even so, it approaches statistical significance by paired experiments (*P*-value, 0.055) and shows excellent formal significance when calculated by paired fish (*P*-value, 0.001).

The fairly large mean difference in survival in homotypic and heterotypic conditioning has a still higher *P*-value (0.088) when calculated with mean results from paired experiments and is not statistically significant. When, however, these last data are calculated as comparisons between paired individuals—with the pairs, of course, established at the beginning of the experiment—the number of pairs becomes 138; the mean difference gives 9.48 per cent larger survival in bullhead-conditioned water; and the *P*-value is 0.008, showing high statistical significance. The individuals surviving for 20 days represented 35, 16, and 10 per cent, respectively, of those originally introduced into bullhead-conditioned, goldfish-conditioned, and control water.

Table 2 shows data similar to those in Table 1, except that they are based on tests with goldfish as assay animals. Although goldfish of the size we used are hardier animals than were the smaller and younger *Ameiurus*, again both types of conditioned water proved superior to

TABLE 1

SURVIVAL OF BULLHEADS

Series	Water	No. of Expts.	No. of Fish	Av. Percentage of Experimental Days Survived	Comparisons	Mean Difference	*P*-Value	
							By Paired Experiments	By Paired Fish
I.....	Goldfish-conditioned	17	195	64.8	I:III.....	4.0	0.055	0.001
II.....	Bullhead-conditioned	17	195	74.7	II:III.....	13.9	0.010	0.0000
III.....	Control	17	195	60.8	II:I.....	9.9	0.088	0.008

the unconditioned medium in permitting fish survival. Here the decrease in toxicity in goldfish-conditioned water is clearly of the same order of magnitude as that in bullhead-conditioned medium. Some additional data concerning survival will be presented in the following section, which deals primarily with growth.

The toxicity of the synthetic pond water made from our once-distilled water during the later months of 1944 and the first half of 1945 was indicated by observations made during the progress of the experiments. Fish were rarely observed to feed in control water after the first week of an experiment, and feces were seldom found in the water. The surviving fish appeared somewhat starved by the end of an experiment. Goldfish in control water often developed a greenish tinge near the head, and bullheads showed a whitish scum over most of their bodies after a few days' exposure to control water (cf. Carpenter, 1927).

The two possible sources of toxicity were (*a*) the distilled water and (*b*) the chemicals, since these, taken together, compose the synthetic pond water. Since the still used is made of copper covered by fused aluminum and since the aluminum lining wears thin with use, copper contamination was suspected. Aluminum poisoning is also a possibility.

Early in 1945, copper was found in the distilled water in a concentration of about 0.04 p.p.m., and approximately 0.08 p.p.m. of aluminum was also present. The analytical methods used may be found in Snell (1937, I, 164 and 265). Even without concentration by boiling, the test for copper has a lower limit of sensitivity of 0.1–1.0 p.p.m.; the aluminum may be estimated in quantities as low as 0.05 p.p.m. Even if the determinations were not quantitatively accurate, we did add at least as much copper and aluminum as occurred in the once-distilled water. Tests of the toxicity for goldfish of these ions in these concentrations gave negative results; copper was introduced as $CuSO_4 \cdot 5H_2O$, and aluminum was added in various forms. There is some question about the accuracy of the copper analysis, and there could be no certainty that the aluminum and copper in our toxicity tests were present in the same form as in the distilled water; hence neither copper nor aluminum can be eliminated completely as the possible toxic agent (see p. 245).

One experiment, in which copper and

TABLE 2

SURVIVAL OF GOLDFISH

SERIES	WATER	NO. OF EXPTS.	NO. OF FISH	AV. PERCENTAGE OF EXPERIMENTAL DAYS SURVIVED	COMPARISONS	MEAN DIFFERENCE	*P*-VALUE	
							By Paired Experiments	By Paired Fish
I.....	Goldfish-conditioned	9	130	87.6	I:III.....	9.4	0.0026	0.0001
II.....	Bullhead-conditioned	8	120	88.8	II:III.....	10.6	0.020	0.0001
III.....	Control	9	130	78.2	II:I.....	1.2	>0.5	0.32

aluminum were introduced simultaneously into double-distilled water, tended to show that the possible synergism between these ions was not in itself responsible for the toxicity of the once-distilled water.

GROWTH ACCELERATION

Too few *Ameiurus* survived the standardized growth period of 20 days to permit analysis of the effect of the two types of conditioned water on growth. However, this study was made with three media, least in the heterotypically conditioned water. There was much individual variation, and the loss is not statistically significant in homotypically or in heterotypically conditioned medium when compared with that shown in unconditioned (control) water. The smaller decrease in weight of fish in the bullhead-conditioned medium has a *P*-value of 0.046, as compared with that shown by their fellows in goldfish-conditioned water.

There are two major reasons for the goldfish, and the results are summarized in Table 3. These data were for the survivors from the same groups of fish whose survival is summarized in Table 2. Growth is shown by changes in weight and in length during 20 days.

TABLE 3

GROWTH OF GOLDFISH

Series	Water	No. of Expts.		No. of Fish		Percentage of Mean Growth		Comparisons	Length		Weight	
		Length	Weight	Length	Weight	Length	Weight		Mean Diff.	*P*-Value	Mean Diff.	*P*-Value
I.....	Goldfish-conditioned	7	6	110	49	0.249	−11.6	I:III...	0.513	0.0000	3.7	0.3
II....	Bullhead-conditioned	7	6	110	46	0.241	− 3.7	II:III..	0.505	0.005	11.8	0.09
III...	Control	8	6	120	41	−0.264	−15.3	I:II...	0.008	>0.5	7.9	0.046

The data on growth generally support those obtained, with survival as the criterion of the suitability of the medium. The goldfish increased in length slightly in both homotypically and heterotypically conditioned water, and they decreased in length in the accompanying controls; both differences have good statistical significance. The slight divergence in growth in length in the two sorts of conditioned water is clearly insignificant.

The assayed goldfish lost weight in all observed differences in rate of growth as determined by weight and length changes, respectively. They are: (1) the experiments from which length measurements of the goldfish are taken do not fully coincide with those in which weight measurements are reported; (2) changes in gut contents or in stored fat affect weights without necessarily and immediately affecting length; hence, in part, the two sets of data measure different processes.

Water that has been conditioned by mud minnows (*U. limi*) also promotes growth of goldfish better than does unconditioned water (Table 4). The lower growth in *Umbra*- as compared with goldfish-conditioned water lacks statis-

tical significance. The survival of the goldfish in this series was rather high throughout, and no appreciable differences exist between the survival of those in conditioned and those in unconditioned water.

In the experiments in which mud minnows were used as experimental animals, no significant differences were found either in growth or in survival between fish that had been in goldfish-conditioned, *Umbra*-conditioned, or untreated water. The growth in control water is, in fact, identical with that in *Umbra*-conditioned water, and the mean of the goldfish-conditioned water is only slightly greater. There is no tendency here or elsewhere toward a detrimental effect of conditioning (Table 5).

Two experiments in which goldfish were tested in *Notropis*-conditioned (one of which measured changes in weight only), goldfish-conditioned, and control water showed no statistically significant differences in growth or survival.

TABLE 4

GROWTH AND SURVIVAL OF GOLDFISH IN GOLDFISH- OR *Umbra*-CONDITIONED WATER

Series	Water	No. of Expts.	No. of Fish	Percentage Mean Growth in Length	Comparisons	Mean Difference	*P*-Value	Percentage Days Survived
I.....	Goldfish-conditioned	6	88	1.35	I:III....	1.61	0.015	99.5
II.....	*Umbra*-conditioned	4	48	0.64	II:III....	0.90	0.013	95.5
III.....	Control	6	120	−0.26	I:II.....	0.71	>0.5	93.8

TABLE 5

GROWTH AND SURVIVAL OF MUD MINNOWS IN HOMOTYPICALLY AND IN HETEROTYPICALLY CONDITIONED WATER

Series	Water	No. of Expts.	No. of Fish	Percentage Mean Growth in Length	Comparisons	Mean Difference	*P*-Value	Percentage Days Survived
I.....	Goldfish-conditioned	7	117	1.43	I:III....	0.23	>0.5	92.7
II.....	*Umbra*-conditioned	7	100	1.20	II:III....	0.0	>0.5	91.4
III.....	Control	7	120	1.20	I:II.....	0.23	>0.5	92.2

SKIN EXTRACTS

The two trials with extracts from the surfaces of bullheads and goldfishes show clear-cut results (Table 6). The differences in growth, when analyzed by paired fish, are quite significant. Both extracts produced accelerated growth in concentrations of 1 : 400,000, and the decrease in length was less in 1 : 800,000 than in the controls. The higher concentration is the more effective, significantly so with goldfish, in which statistical probability of the increased growth is 0.0014.

The extracts from two species of fishes gave essentially similar results when they were used, as they were in these tests, in similar concentrations. Perhaps this indication should be accepted with some reservation pending the accumulation of more data.

DOUBLE-DISTILLED WATER

The previous discussion has been limited to results obtained while using

once-distilled water. Enough trials have been made to demonstrate that the situation is different when the artificial pond water is made from twice-distilled water. All the assay fishes lived throughout the experiments in all the kinds of media tested when this was based on redistilled water. Positive growth also was given in control, as well as in conditioned, water. Hence the toxic agent was introduced into the synthetic pond water by the once-distilled water, not by the chemicals or by other sources of possible contamination. A trace of copper was found in one lot of redistilled water; although the concentration was below the limit of sensitivity for the quantitative method, the amount of copper present was apparently less than in our water before redistillation. The growth of goldfish in either kind of conditioned water did not differ significantly from that in the unconditioned controls; the *P*-value is 0.5 in both comparisons. There was a suggestion that bullhead-conditioned water might be a more favorable medium for goldfish growth than was homotypically conditioned water.

TABLE 6

GROWTH AND SURVIVAL OF GOLDFISH IN FISH EXTRACTS

Series	Extract	Concentration	No. of Expts.	No. of Fish	Percentage Days Survived	Percentage Mean Growth in Length	Comparisons of Growth			
							Series	Difference	No. of Pairs	*P*-Value
I.....	Goldfish	1:400,000	2	48	99.6	+0.40	I:V.....	2.88	16	0.0002
II.....	Goldfish	1:800,000	2	48	100	−0.73	II:VI....	1.38	15	0.0210
III.....	Bullhead	1:400,000	1	24	100	+0.56	III:V....	3.04	9	0.0008
IV.....	Bullhead	1:800,000	2	48	100	−0.33	IV:VI...	1.78	15	0.0030
V.....	Controls for I and III	.....	2	36	77.5	−2.48	I:III....	0.16	23	>0.47
VI.....	Controls for II and IV	.....	2	36	76.7	−2.11	II:IV....	0.40	48	>0.50
							I:II.....	1.13	46	0.0014
							III:IV...	0.89	21	0.11

THE CONDITIONING COEFFICIENT

Allee *et al.* (1936) introduced the practice of using a so-called conditioning coefficient as a rough indication of the degree of contamination of the raw synthetic pond water brought about by conditioning. It may be remembered that the fishes are not fed in the water that they are conditioning and that all changes in the medium, over and above those found in the controls, are produced directly by the contained fishes.

The conditioning coefficient is obtained by dividing the sum of the lengths of the conditioning fish in millimeters by the liters of water contaminated during 22 hours. This value was kept close to 25 during the present experiments. The conditioning coefficient gives only a rough indication at best and is less useful in heterotypic than in homotypic comparisons. Fishes of different species vary decidedly in body surface, in slime secretion, and in food regurgitation, even when otherwise somewhat comparable. Two methods were used to find some indication of the amount of variation in conditioning by certain of the species used under different conditions of nutrition.

Amino nitrogen.—Amino nitrogen was determined by means of the Kjeldahl method (Ma and Zuazaga, 1942). Nine or ten fish of approximately 50-mm. length were placed in 1 liter of synthetic pond water. The individuals were selected so that the total length of the fish for each liter of water was about 500 mm. They were fed—or starved—for varying lengths of time, depending on the experiment, and were then placed in clean water. This water was conditioned for 22 hours; the fish were removed. The water was transferred to a volumetric flask and made up to 1 liter in each case. Of this water, 500 cc. were evaporated on the steam bath to a volume between 10 and 20 cc., which was transferred to 25-cc. volumetric flasks and made up to the mark. The conditioning coefficient of this water is 10,000. For the actual determinations, 2 cc. of water were pipetted directly into the digestion flasks and analyses were made on these samples. The results are summarized in Table 7.

It is evident that there may be a considerable variation between the amount of amino nitrogen in successive tests under similar conditions but with different conditioning fish. The bullheads introduced consistently less amino nitrogen into the water than did goldfish; mud minnows were intermediate in this respect. It should be noted that goldfish are a deeper-bodied fish than are either of the other species tested, and hence, given the same total length, they expose more surface to the surrounding water; this may or may not have been the basis of the difference in amino nitrogen.

TABLE 7

KJELDAHL DETERMINATION OF AMINO NITROGEN IN WATER WITH A CONDITIONING COEFFICIENT OF 10,000

(All Results in Parts per Million)

Series	Species	Length (in Mm.)	Diet		
			Soybean and corn meal	Liver	Starved
I (February, 1945)	Goldfish	55–60	99	282	238*
		40–45	111	270	226*
	Bullheads	45	70	197	186*
		30	58	175	146*
II (November, 1945)	Goldfish	48	97	198	94†
	Mud minnows	56	84	186	85†
	Bullheads	39	49	88	49†

* Starved 2 days after feeding liver.

† Starved 5 days after feeding liver.

Required oxygen.—Required oxygen depends on the amount of organic material present and is expressed in terms of the amount of oxygen needed to oxidize such material to a fairly stable end-product. The method used is described in the *Official and Tentative Methods of Analysis* of the Association of Official Agricultural Chemists (1935, p. 510). The period of boiling with permanganate was limited to 10 minutes with water with a

conditioning coefficient of 250, ten times that used in experimental assays of growth and survival. The same method was used by Allee, *et al.* (1936), except that we have introduced a correction for sulphides, nitrites, and ferrous salts by letting potassium permanganate react with the heavily conditioned medium for 3 minutes at room temperature. The results summarized in Table 8 are indicative rather than definitive. The effect of introduced by the bullheads (Table 7), the indications are that there is no essential difference in the oxidizable organic material of these media when, as was usually the case, the conditioning fishes were fed the soy- and corn-meal mixture.

DISCUSSION

There is growing evidence that, under many experimental conditions, various

TABLE 8

REQUIRED OXYGEN IN WATER WITH A CONDITIONING COEFFICIENT OF 250

(All Results Are in Milligrams)

Series	Fish	Mean Length (Mm.)	Diet			
			Cereals	Liver	Starved	Control
I Allee *et al.* (1940, p. 428)	Goldfish	.....	5.11	11.21	2.90	0.0
II (December, 1945)	Goldfish	40				
	Gross	.....	7.01	9.80	3.52	0.2
	Correction	.....	1.25	1.27	1.03	.....
	Net	.....	5.76	8.53	2.49	.....
	Bullheads	45				
	Gross	.....	7.47	8.89	5.16	.....
	Correction	.....	1.54	1.25	1.39	.....
	Net	.....	5.93	7.64	3.77	.....

starvation and of type of food is consistent in all determinations. Starved fishes introduced the smallest amount of oxidizable organic material; those previously fed on liver produced the most.

The observed differences between water conditioned by these two species of fishes are smaller than are those between determinations made on goldfish-conditioned water in 1940 and again in 1945–46. Furthermore, the variations between goldfish- and bullhead-conditioned media are not consistent under the tested conditions of nutrition. Unlike the smaller amount of amino nitrogen aquatic animals thrive better in a slightly contaminated medium than in a wholly uncontaminated one, although not all animals benefit from such exposure (Allee, 1931; Park, 1936). For fishes, growth acceleration in homotypically conditioned medium has been reported from this laboratory by Church (1927), Shaw (1932), and Allee, *et al.* (1934), using *Platypoecilus maculatus;* by Allee *et al.* (1934) using *A. melas;* and by Allee *et al.* (1934, 1936, 1940), Evans (1936), and Livengood (1937), using *C. auratus.*

Growth acceleration has been obtained by homotypic conditioning in the follow-

ing types of water: Whitman Laboratory well water, *Platypoecilus, Ameiurus;* filtered, chlorinated water from Lake Michigan, *Ameiurus;* synthetic pond water (p. 245), *Carassius.*

No effect of conditioning was found by Allee *et al.* (1940) in the following tests: (1) synthetic pond water made up in double-distilled water—one experiment; (2) charcoal-filtered, Chicago tap water (originally from Lake Michigan)—three experiments by Finkel; (3) untreated water directly from Lake Michigan—one experiment; (4) dilute Ringer's solution —two experiments; (5) a water made from distilled water to approach the supposed ionic composition of Lake Michigan—two experiments. It is necessary to state that in seven of the above-mentioned experiments in which tests were made simultaneously in the usual synthetic pond water there was also an insignificant difference, for unknown reasons, between growth in conditioned and in control water. Also the fish grew better in synthetic pond water made in bi-distilled water than in accompanying tests in a similar medium made in once-distilled water soon after the steam still had been lined with fused aluminum.

Considering results from what may be called the "standard" tests using once-distilled water, there were forty-six experiments available for analysis in 1940, each of which had lasted from 20 to 30 days. In all, 686 goldfish from homotypically conditioned water grew 0.47 units more than did 614 accompanying controls. The difference is highly significant statistically, with a *P*-value of 2×10^{-7}.

As recognized at that time (Allee *et al.*, 1940, p. 425), further supporting evidence for this species in this kind of medium is not needed; yet we have incidentally collected supporting evidence in the present series of experiments. Goldfish grew in length significantly more rapidly in homotypically conditioned, once-distilled water, as compared with accompanying controls. In seven experiments (Table 3), in six more (Table 4), and in one other comparable test, a total of 207 fish were assayed in the goldfish-conditioned water and 249 in controls; the goldfish grew, on the average, 0.28 units more in the conditioned medium. The *P*-value of the difference, calculated by using paired experiments, is 0.04.

As in other years, some experiments departed from the general trend. For example, we failed to find the usual size-conserving, or growth-promoting effect in the two experiments that constitute another series (p. 250) and again in two tests in which the standard artificial pond water was based on twice-distilled water.

The growth-accelerating power of the conditioned water results (*a*) from the presence of particles of regurgitated food introduced by the conditioning fishes and taken up by the assay animals (Allee *et al.*, 1936; Livengood, 1937); (*b*) by the introduction of a growth-promoting substance (Allee *et al.*, 1936, 1940); and (*c*) by the adsorption or other fixation of some toxic substance. This latter possibility has been considered repeatedly. Although Allee *et al.* (1940) did not regard it as being effective in the experiments which they conducted, lessened toxicity appears to be a significant factor in the present series of tests. In several of the experiments, especially those with quite young bullheads, the untreated control water was especially toxic, and certain of the results summarized in Tables 1 and 2 deal primarily with differential survival rather than with differential growth. Better growth and sur-

vival occurred in bi-distilled water (from glass or tin-lined stills), again demonstrating the removal, or partial removal, of some toxic material. Experiments have not been made to test the relative effect on growth of water redistilled in tin and in glass (cf. Finkel and Allee, 1940).

The results from experiments with dilute solutions of skin extracts (Table 6), in addition to confirming previous results (Allee *et al.*, 1936, 1940), throw light on the relations between differential toxicity, survival, and growth. Both goldfish and bullheads survived much better in once-distilled water containing skin extracts than they did in the untreated, control water. The full effect on survival was produced by the weaker concentration; hence the acceleration in growth of goldfishes at a concentration of 1 : 400,000, as contrasted with a slight decrease in length in a concentration of 1 : 800,000, resulted either from a further decrease in toxicity over and above that required to prevent deaths or from a positive growth accelerator, such as was assumed to be operating in earlier reports from this laboratory on this subject. Perhaps, as often happens, both sets of factors were acting together.

All the species of fishes used in the present series of experiments are relatively or quite hardy, young of *A. melas* from the juvenile swarming stage being least so. Perhaps their marked hardiness is one reason why the mud minnows which were tested showed no effect of conditioning either on growth or survival. The negative results with *N. cornutus* must be dismissed for the moment, at least, as inconclusive because of the few tests. Experiments of the kind we have been making require many repetitions to establish a given result with certainty. (Probably all these experiments should be repeated with more sensitive species.)

The discussion thus far has dealt with confirmations and a slight extension of previous knowledge concerning homotypic conditioning. Newer ground is broken by the comparisons between the relative potency of homotypic and of heterotypic conditioning of water by some of these species. The effect on toxicity can be dismissed with a few words. The results obtained indicate little difference in protective power in the two types of biologically treated water. This is according to expectation if the conditioning fishes, directly or indirectly, serve to fix some toxic agent that may be present, perhaps in the form of metallic ions. Certainly, there was no difference in protection by the skin extracts (Table 6) when these were introduced in equal amounts of dry weight. Again this is according to expectation, provided, as was true in these cases, that neither extract is noticeably toxic on its own account.

There is no indication of differential effects of homotypic, as compared with heterotypic, conditioning in the survival of goldfish, whether in goldfish- as compared with bullhead-conditioned water or in goldfish- in comparison with *Umbra*-conditioned medium. There is a suggestion that bullheads survived better in homotypically conditioned water than in goldfish-conditioned medium. The evidence that bullhead-conditioned water allowed goldfishes to maintain weight better than did homotypically conditioned water is somewhat better but must be balanced against the fact that the difference in growth in length in the two waters is insignificant.

The better survival of bullheads and the better maintenance of weight by goldfishes, together with a suggestion in

the same direction from the increased growth in bullhead-conditioned media made with bi-distilled water, all suggest some slight superiority of bullhead- over goldfish-conditioning. This possibility should be considered in the light of the results from Kjeldahl determinations of amino nitrogen and from the amount of organic matter present as indicated by required oxygen. The bullheads introduced decidedly less amino nitrogen than did the comparable goldfishes under the conditions tested, but they apparently introduced about the same amount of oxidizable organic material.

Since bullhead-conditioned water is only a slightly, if any, better medium for survival or growth of these fishes than is water modified by goldfish conditioning, the observed effects would seem to be more closely related to the amount of oxidizable organic matter present than to amino nitrogen. To the extent that this is true, the indications from measurement of required oxygen make it highly probable that changing the food of the conditioning fish may well introduce more important variations in the conditioned water than are brought about by changing from goldfishes to bullheads as conditioning agents.

In the present state of our knowledge, high probability exists at only one point in this phase of the discussion: similar effects on growth and survival were produced by the introduction of equal amounts of extracts from the surfaces of goldfishes and of bullheads. This indicates that, given equal quantities of skin secretions and in so far as these control the conditioning processes, these two species approach equality as conditioning agents.

It is quite probable that the protective action of the conditioned media and perhaps some of its growth-promoting power, too, result from adsorption. If so, subtle surface characteristics of adsorbing particles could easily be the effective mechanisms in conditioning, and these properties would not be revealed by any of the analyses we have attempted.

Again it is worth noting that there has been no indication during the present series of experiments of a final deleterious effect of any kind produced by these strengths of conditioned media. This supports previous experience in our laboratory. Fish growth is definitely retarded in more strongly contaminated water.

The marked similarity of effects produced by homotypic and heterotypic conditioning upon survival of *Ameiurus* and *Carassius* and upon the growth of the latter suggests either that such differences as exist in the two types of conditioned media are not important with fishes, that different mechanisms produce similar effects, or that the finer distinctions of the effects of conditioning await an assay with more sensitive species of fish.

Certain of the broader implications of the protective and accelerating action of conditioned media on many organisms have been presented repeatedly by the senior author, especially in 1931, 1938, 1943, and 1945, and do not need restatement here.

SUMMARY

1. Young *A. melas* show better survival in an artificial pond water made in once-distilled water when this medium has been conditioned by having *Carassius* or other *Ameiurus* live in it than they do in unconditioned (control) water.

2. The homotypically conditioned medium allows slightly longer survival for *Ameiurus* than does the heterotypically conditioned one tested.

3. Similarly, young *C. auratus* survive longer in these conditioned media than in the uncontaminated controls, except that with goldfish the two types of water were equally effective in promoting survival.

4. Although introduced copper and aluminum ions, in concentrations comparable with those found in our once-distilled water, produced no deaths, copper contamination of the water is suspected of being the toxic agent, the activity of which is greatly decreased or nullified by conditioning. Aluminum in some form may also be related to the observed toxicity. Very low concentrations of both metallic ions were present in the once-distilled water.

5. An otherwise similar artificial pond water, based on water redistilled in a tin-lined still, was not toxic, and positive growth was given by assay fish, even in the control medium.

6. As in earlier experiments, goldfish grew in length more rapidly in the conditioned water than in uncontaminated, but otherwise similar, control media. They maintained weight slightly better in bullhead- than in goldfish-conditioned water.

7. Extracts from the surfaces of bullheads and of goldfishes have essentially similar protective and growth-promoting powers when used in equal concentrations.

8. Water with the same conditioning coefficients contained less amino nitrogen following bullhead- as contrasted with goldfish-conditioning. Differences in oxidizable organic matter in the two types of conditioned media are smaller and less consistent than are the variations produced by changing the diet of the conditioning fish.

9. With the concentrations used in these experiments, all observed effects of conditioned water were, on the average, either neutral or favorable in their effects on fish survival, weight maintenance, or growth. There is a brief discussion of causal factors.

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ENDOCRINE FACTORS CONTROLLING ERYTHROCYTE CONCENTRATION IN THE BLOOD OF THE DOMESTIC FOWL[1]

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EVIDENCE has been accumulating which indicates that the concentration of circulating erythrocytes is in part controlled by endocrine factors; and hormones from the ovaries, testes, pituitary, thyroids, and adrenal cortex have been reported as partly or entirely responsible for such control. It has been postulated that these hormones may act (1) directly, by stimulating or inhibiting the production of red blood cells in the bone marrow; (2) indirectly, by producing general metabolic changes; or (3) by bringing about shifts in tissue and circulating fluids, thereby affecting the concentration of erythrocytes in the blood but not their absolute numbers.

[1] This investigation was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. We are indebted to Professor Sewall Wright for advice concerning statistical methods; to Professor William Bloom and Dr. Leon O. Jacobson for their critical reading of the paper in manuscript; and to Mr. Ben B. Blivaiss for putting his valuable thyroidectomized birds at our disposal, for contributing data on comb growth, and for other helpful assistance. All this aid is gratefully acknowledged.

Since a sex difference in the number of red blood cells has been definitely established in the domestic fowl, as in many other species, it seemed plausible to assume that the sex hormones might be an important contributing factor in their control. In previous papers (Domm, Taber, and Davis, 1943; Taber, Davis, and Domm, 1943) we have presented evidence to show that the erythrocyte count of capons dropped to the female level and was essentially the same as that of bilaterally ovariectomized poulards, while the count of sinistrally ovariectomized poulards was between those of normal males and normal females. It was concluded that the development of a right ovotestis, with its subsequent secretion of androgen as well as estrogen, was responsible for this increase above the normal female level. The fact that the concentration in such individuals did not reach the male level might be attributed to an inadequate androgen supply (Davis and Domm, 1943) or to the presence of a mixture of androgen and estrogen. Continued obser-

vations on the red-cell counts of a large number of normal and operated birds have confirmed these results. The average counts for untreated cocks, hens, capons, and sinistrally and bilaterally ovariectomized poulards and the number of counts from which these averages were obtained are given in Table 1. It was further shown that male sex hormone injected into hens or capons caused a rise in the number of erythrocytes, while female sex hormone injected into roosters or capons had the reverse effect.

The observations reported in this paper are a continuation of earlier experiments (Domm, Taber, and Davis, 1943; Taber, Davis, and Domm, 1943) which were undertaken to add additional data to those already obtained on the effects of androgens and estrogens, injected or administered in pellet form, on the red-cell counts of the domestic fowl. An attempt was made to determine whether there existed a consistent and substantial variation in the counts of normal and operated birds during different seasons of the year or at different times of the day and night. In addition, observations were made on thyroidectomized birds and on roosters, hens, and capons treated with thiouracil. Preliminary reports of some phases of this work have already been published (Taber and Domm, 1942, 1943, 1944).

MATERIALS AND METHODS

All the birds used in these experiments were of the single-comb, light-brown Leghorn variety and, with the exception of three groups of pullets, were mature individuals. The capons, poulards, and thyroidectomized birds had been operated upon during the first month after hatching, and feather and comb characteristics at the time counts were made indicated that all operations were complete.

The relatively large ulnar vein lies immediately beneath the skin on the inner surface of the elbow joint. By pricking this vein with a sharp needle, we could easily obtain a momentary flow of several drops of blood, which was usually followed by little or no further hemorrhage. The blood was drawn directly into a standard dilution pipette and was diluted with a solution of 1.5 per cent sodium citrate and 0.85 per cent sodium chloride. An improved hemocytometer with a double Neubauer counting chamber was used for all counts. When counts were made frequently on the same birds, the right and the left wings were used alternately. Counts which were exceptionally high or low were rechecked before being included.

TABLE 1

ERYTHROCYTE COUNTS IN UNINJECTED BIRDS

Sex	No. of Birds	No. of Counts	Av. Red Cell Count*	Standard Deviation
Male	82	288	3.25	.434
Female	84	678	2.61	.384
Capon	89	401	2.48	.295
Sinistrally ovariectomized poulard	30	134	3.00	.331
Bilaterally ovariectomized poulard	16	67	2.60	.257

* Expressed in millions per cubic millimeter.

In order to determine whether the distribution of erythrocytes was random or whether a significant amount of clumping occurred, a series of thirty-one counts was made, with the methods employed throughout these experiments. Each sample included counts made from five squares extending diagonally across the counting chamber. The chi-square dispersion $(\Sigma[V - \bar{V}]^2/\bar{V})$ was calculated for the five counts in each sample, and the average, 3.54, was close to the

theoretical value, 3.36, for probability .50 and 4 degrees of freedom. The distribution of values agreed well with that expected ($X^2 = 2.71$; $N = 5$; Probability = .70–.80).

Observations were made at intervals of 2–7 days at the beginning of experiments and then at wider intervals when observations were continued over a long period. In all cases control counts were made on untreated birds. No attempt was made to replace treated birds that died during the course of long-term experiments. Comb measurements were made weekly on many of the treated and control birds. These measurements are expressed in the graphs as the product of height and length.

Testosterone propionate, alpha-estradiol benzoate, and diethylstilbestrol were administered either by means of daily intramuscular injections or by subcutaneous pellet implantations. Subsequent implants were made when palpation indicated that the pellets were absorbed. Thiouracil in measured amounts was administered daily as a dry powder in a gelatin capsule, which was placed on the back of the tongue and palpated down the esophagus. The exact amounts of each hormone used and the duration of treatment are included in the discussion of results.[2]

RESULTS

1. ANDROGEN INJECTED CAPONS

Although it had been definitely established that daily injections of testosterone propionate would raise the erythrocyte number of hens and capons, no counts had previously been made at short-enough intervals to tell exactly when the effect could first be observed and how long after cessation of injections the effect was maintained. Consequently, four capons were injected with 1 mg. testosterone propionate daily for 72 days, and red-cell counts were made at intervals of 2–4 days throughout the injection period and for 40 days after cessation of injections. The first definite increase in the number of erythrocytes was observed on the tenth day, and the average male level was reached on the eighteenth day. The counts fluctuated slightly above or below that point for the duration of the injection period and for 16 days after injections ceased. The highest average count, 4,260,000 red cells per cubic millimeter, was observed on the fortieth day of injections. By the eighteenth day after injections ceased, a definite fall was observed, and the counts had returned to the normal capon level by the twenty-eighth day (see Fig. 1).[3]

The capon is particularly well adapted for such a study, since the comb is an excellent indicator of the presence of effective amounts of male hormone. The increase in comb size and the increase in erythrocytes showed a direct correlation, except at the beginning and at the end of the injection period: the comb response was immediate, while there was a short lag in the rise and fall of the erythrocyte number.

2. PULLETS IMPLANTED WITH PELLETS OF ANDROGEN

a) Five months old.—Four pullets were implanted with pellets of testosterone propionate, averaging 44.2 mg. (range,

[2] Grateful acknowledgment is made to Dr. Erwin Schwenk, of the Shering Corporation, for the testosterone propionate (Oreton) and alpha-estradiol benzoate (Progynon-B); to Dr. J. A. Morrell, of E. R. Squibb & Sons, for the diethylstilbestrol; and to Dr. George F. Cartland and Dr. M. H. Kuizenga, of the Upjohn Company, for the thiouracil used in these experiments.

[3] It is possible that experimental procedures similar to these may prove valuable in determining the life-span of red blood cells, as well as the time required for their maturation.

40.4–46.8); two controls were given similar pellets of cholesterol. The first noticeable rise in the number of erythrocytes was observed on the tenth day, at which time the male level had been attained. During the following 20 days the counts continued to rise, reaching a high peak of 3,900,000 red cells per cubic millimeter on the thirtieth day. Following this, there was a gradual fall in the number of erythrocytes until the control level was reached on the fifty-fourth day. The counts fluctuated around the female level until after the eighty-fourth day, at which time a second pellet (av. weight, 31.4 mg.; range, 30.6–31.8) was implanted in each bird. Six days after the second pellet implantation, a rise in the number of erythrocytes was observed, and the male level was reached by the fourteenth day. Although the counts never reached the high peak observed previously, they were maintained above the controls and within the male range for 52 days, then fell again to control level. Subsequent implants were made on the 164th, 244th, 336th, 424th, 530th, and 630th days (av. weight of pellets, 29.0 mg.; range, 28.0–30.2), and regular observations were continued for a period of 274 days, followed by monthly or bimonthly counts for 416 additional days. After each implant there was the same characteristic rise in the number of erythrocytes, followed by a return to or near the control level when the pellets were almost or entirely absorbed; but rarely did the counts reach the high peak or subsequently fall as abruptly as observed during the first period. Figure 2 graphically illustrates the red-cell counts and comb measurements for 522 days, including six implantation periods.

It is interesting to observe that the close correlation between the amount of comb growth and the increase in the number of red blood cells shown in the testosterone-injected capons was not observed in the pellet-implanted pullet series. This was especially true during the first implantation period (see Fig. 2). While the blood counts of both groups fluctuated between 3,300,000 and 3,700,000, the greatest comb development during this period was 9.2 × 5.3 cm.[4] in the treated pullets, as compared with 14.8 × 8.8 cm. in the injected capons. During the entire course of observations on the pullets, the greatest growth of the comb was 12.6 × 7.7 cm., noted on the 476th day, during the sixth implantation

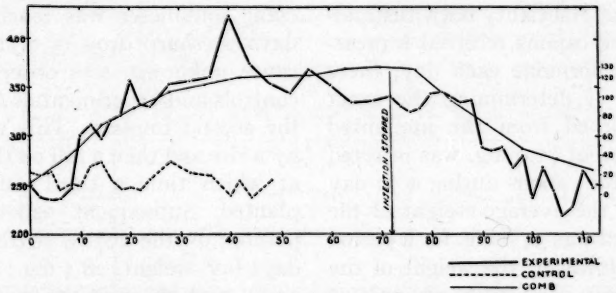

FIG. 1.—Graphs illustrating erythrocyte counts and comb size in capons injected with 1.0 mg. testosterone daily for 72 days. (*Left ordinate*, erythrocytes in millions per cubic millimeter; *right ordinate*, product of comb length and height expressed in centimeters; *abscissa*, number of days after initiation of treatment.)

[4] Comb length *times* height.

period. Control combs measured 7.4 × 4.3 cm. at this time. The reasons for this difference in response are not clear, but several possible contributing factors might be mentioned. In the pullet group, during the first implantation period, we were dealing with immature birds which had not attained full body or comb growth. Their combs measured 4.4 × 1.3 cm. at the beginning of the experiment, while the capons measured 5.5 × 2.4 cm. We were concerned with females in one group and castrated males in the other, and genetic factors may be involved, the theory of equipotentiality notwithstanding. While the capons received a measured dose of hormone each day, there was no way of determining the exact amount absorbed from the implanted pellets. A total of 72.0 mg. was injected into each of the capons during a 72-day period, while the average weight of the pellets per bird was 34.8 mg. for a period of 84 days. However, the weight of the capons (1,980–2,047 gm.) was about twice the weight of the pullets (889–1,160 gm.). It is possible, nevertheless, that the rate of absorption from the pellets was below the level for maximum comb response but adequate to cause a rise in the red blood cells to the male level.

It was also observed that, while the combs of the capons regressed to their previous size at the termination of injections, there was little or no comb regression in the pullet group between pellet implantations. Here again the normal maturation processes in the pullets, including comb growth (note comb growth in controls, Fig. 2) must be considered; and endogenous androgen, known to control comb growth in hens, might be sufficient to maintain the increased comb size until the subsequent implantation.

b) One month old.—In order to determine if the increased erythrocyte response could be elicited in younger pullets, six 1-month-old pullets were implanted with pellets of testosterone propionate averaging 14.3 mg. (range 14.0–14.6). Three controls received similar implants of cholesterol. There was little change in the red-cell count for 109 days, at which time a second pellet was implanted (av. weight, 26.6 mg.; range 25.0–28.2). After 11 days a pronounced increase in the number of erythrocytes was observed, and the male level was attained. The counts continued to increase until a peak of 3,550,000 red cells per cubic millimeter was reached after 41 days. A sharp drop in erythrocytes, its cause unknown, was observed in both controls and experimentals 63 days after the second implant. This was followed by a rise and then a fall on the 202d day, at which time a third pellet was implanted. Subsequent pellets were implanted on the 297th, 395th, and 495th days (av. weight, 28.3 mg.; range, 26.2–30.0), and observations were continued through 557 days. During this period the red-cell counts of treated birds were maintained above the level of the controls.

Comb stimulation was observed 13 days after the first pellet implant. There was a slow but consistent increase in size throughout the course of observations (399 days), with little or no indication of regression when the pellets approached absorption. In both controls and treated birds the period of greatest comb growth was during the fifth month of age. Figure 3 shows the red-cell counts and comb measurements through 250 days.

The complete lack of erythrocyte response shown by the pullets during the first implantation period could be due to several factors. If testosterone propionate produces its effects by stimulating erythropoiesis, it is possible that the mar-

row cells are not responsive at this early age. Or it is quite possible that the small size of the first pellets implanted, providing less surface for absorption of the hormone, maintained a hormone level in the blood below the threshold necessary for the stimulation of red-cell production. This was not considered likely, however, since the size of the pellets was, in proportion to body weight (156 gm.), much greater than in the 5-months-old series.

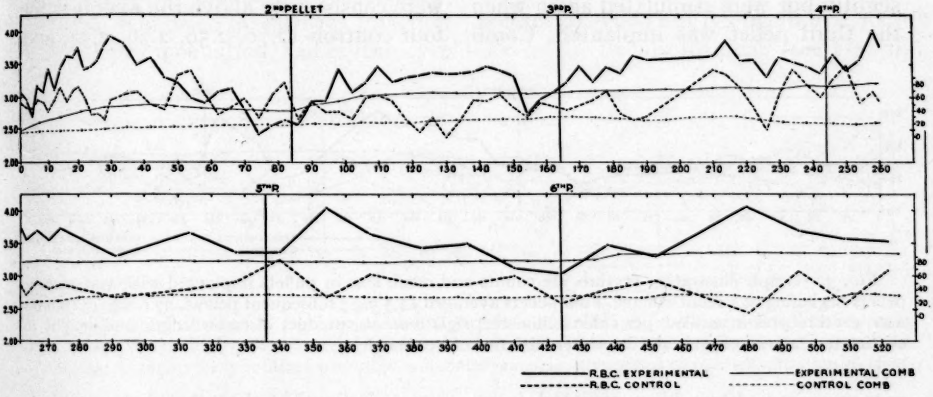

FIG. 2.—Graph illustrating erythrocyte counts and comb size in pullets implanted with testosterone pellets beginning at 5 months of age. First pellets averaged 44.3 mg.; subsequent pellets, 29.3 mg. (*Left ordinate*, erythrocytes in millions per cubic millimeter; *right ordinate*, product of comb length and height in centimeters; *abscissa*, number of days after initiation of treatment.)

c) Three months old.—Two groups, consisting of three pullets each, were implanted with single pellets of testosterone propionate averaging 26.8 mg. (range, 24.6–28.8 mg.), and three controls received cholesterol pellets. A rise in the number of erythrocytes was observed on the sixth day in both experimental groups. For 66 days the counts fluctuated between 2,800,000 and 3,250,000 erythrocytes per cubic millimeter, with an average level of about 3,000,000, as compared with 2,750,000 in the controls. On the eightieth day the pullets in one group were given two pellets each (av. weight, 56.3 mg.; range, 56.0–56.8 mg.), while those of the second group received only one pellet (av. weight, 28.7 mg.; range, 28.2–29.2 mg.). Following these implantations the red-cell count rose in both groups. During the first 35 days the counts of pullets with two pellets fluctuated between 3,250,000 and 3,500,000, while the counts of the other group fluctuated from 2,600,000 to 3,500,000. For the remainder of the period, counts of both groups fluctuated around 3,000,000. Of the seventeen counts made during the entire period, twelve were higher in the birds with the double-pellet implants. One bird of each experimental group died during these observations; therefore, on the 185th day, only two birds were given double pellets (av. weight, 57.7 mg.; range, 57.4–58.0 mg.), and two received single pellets (av. weight, 28.5 mg.; range, 28.2–28.8 mg.). The pullets that received two pellets showed a steady rise in erythrocytes, reaching a peak of 3,830,000 per cubic millimeter. The counts of those receiving

only one pellet were lower, reaching a peak of 3,350,000, but they maintained an average above the controls for the greater part of the period (see Fig. 4).

Comb stimulation could be observed in both experimental groups within 1 week of the first pellet implantation. The pullets which subsequently received double implants showed no comb regression toward the end of the second implantation period, while the combs of those receiving only a single pellet regressed slightly but were stimulated again when the third pellet was implanted. Comb measurements have been omitted from Figure 4 to simplify the graph. In general, the picture is the same as in Figures 2 and 3.

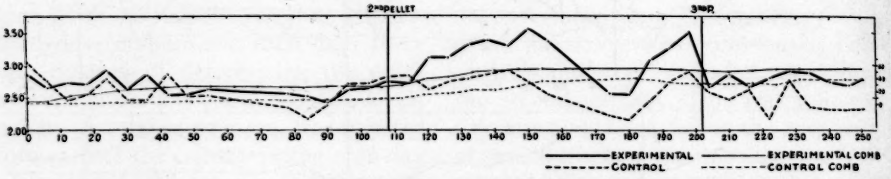

FIG. 3.—Graph illustrating erythrocyte counts and comb size in pullets implanted with testosterone pellets beginning at 1 month of age. First pellets averaged 14.3 mg.; subsequent pellets, 27.6 mg. (*Left ordinate*, erythrocytes in millions per cubic millimeter; *right ordinate*, product of comb length and height in centimeters; *abscissa*, number of days after initiation of treatment.)

The fact that the hens with two pellets showed higher erythrocyte counts than those with a single pellet would seem to indicate that the number of red blood cells is, within limits, dependent upon the amount of male hormone available and that the lack of response in the 1-month-old pullets may have been due to an inadequate amount of hormone. Our observations on the 5-months-old pullet series also substantiate this argument. These pullets showed a greater erythrocyte response during the first pellet dynasty, when the pellets averaged 44.3 mg., than during subsequent periods, when the average pellet weight was 29.3 mg. (see Fig. 2). Further evidence for this interpretation has been obtained from observations now in progress, which will be reported in greater detail at a later date. Eight female chicks were given pellets averaging 13.8 mg. on the third day after hatching. No erythrocyte counts were made prior to the thirtieth day, but counts made at weekly intervals from the thirtieth to the sixtieth day, although below the male level (2.92, 2.75, 2.74, 2.56, and 2.81 million red cells per cubic millimeter), were consistently above the averages for four controls (2.70, 2.59, 2.36, 2.54, and 2.57), indicating that these young birds did not lack the ability to respond.

However, after a second implantation, on the sixtieth day, when the weight of the pellet was doubled (27.6 mg.), the erythrocyte picture was similar to that observed during the first pellet dynasty, and a rise to the male level was not observed until after a third implantation on the 176th day. Since a pellet weighing 27.6 mg. elicited no greater response in birds 60 days old than a 13.8-mg. pellet in birds 3 days old and, since a 28.3-mg. pellet was sufficient to raise the erythrocyte count to the male level in birds 176 days old, the importance of age as a factor cannot be dismissed. Furthermore, in neither the 3-months-old nor the 1-month-old series have we observed average erythrocyte counts at or above the

male average until during the sixth month of age. In view of this evidence, one is led to believe that, although the erythrogenic tissue in young pullets is capable of responding to hormone stimulation, the degree of response is not so great as in hens which have attained the age of sexual maturity.

3. SIMULTANEOUS TREATMENT WITH ANDROGENS AND ESTROGENS

a) Daily injections in capons.—We have previously reported that intersexual males, feminized by estrogen injection during early incubation, had erythrocyte counts lower than normal males but above the female level (Taber, Davis, and Domm, 1943). In an attempt to duplicate this hormonal condition, thirteen capons were given daily injections of 1.0 mg. of testosterone propionate for 25–35 days. In addition to the testosterone, three of them received 0.33 mg. of alpha-estradiol benzoate daily, three received 0.75 mg. of alpha-estradiol benzoate, and three received 1.0 mg. of diethylstilbestrol; the remaining four testosterone-injected capons served as controls. Three other capons received daily injections of 0.33 mg. of alpha-estradiol alone. Counts were made at 10- or 15-day intervals.

The red-cell counts of capons treated with testosterone alone rose from 2,510,000 to 3,610,000 erythrocytes per cubic millimeter, while the counts of the three capons treated with alpha-estradiol alone dropped from 2,620,000 to 2,180,000 during the injection period. The capons with testosterone plus 0.33 mg. of alpha-estradiol paralleled the testosterone-treated birds, while those receiving testosterone plus 0.75 mg. of alpha-estradiol fell from 2,340,000 to 2,090,000 and then rose again to 2,610,000; those receiving testosterone plus stilbestrol rose from 2,780,000 to 3,010,000 but fell again to 2,790,000 on the twenty-fifth day (see Fig. 5).

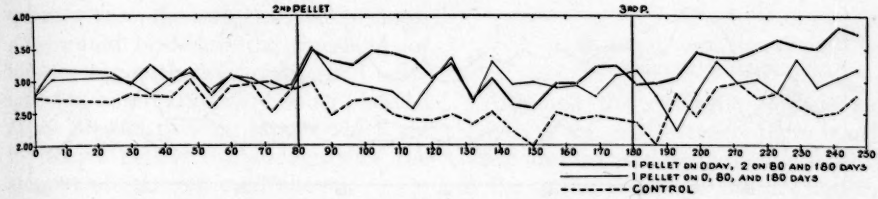

FIG. 4.—Graph illustrating erythrocyte counts of pullets implanted with testosterone pellets beginning at 3 months of age. First pellets averaged 26.8 mg.; subsequent single pellets, 28.6; double pellets, 57.0 mg. (*Ordinate*, erythrocytes in millions per cubic millimeter; *abscissa*, number of days after initiation of treatment.)

It would seem from this that small doses of estrogen combined with testosterone have no inhibitory effect on the red-blood-cell count, but that the stimulating effect

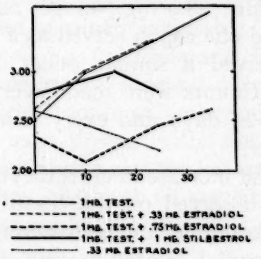

FIG. 5.—Graph illustrating erythrocyte counts of capons injected with androgen only, estrogen only, and with both androgen and estrogen. (*Ordinate*, erythrocytes in millions per cubic millimeter; *abscissa*, number of days after initiation of treatment.)

of testosterone may be counteracted by larger doses of estrogen.

b) Pellet implants in capons.—Since observations on the doubly injected capons in the series above were made at relatively widely spaced intervals, it seemed desirable to obtain a more complete record of the red-cell picture in capons treated simultaneously with androgens and estrogens. Consequently, three capons were given testosterone propionate pellets averaging 29.3 mg. (range, 29.0–29.4 mg.), three capons were given pellets of alpha-estradiol benzoate averaging 26.8 mg. (range, 26.4–27.6 mg.), three capons were given alpha-estradiol pellets (av. weight 28.4 mg.; range, 28.0–29.0 mg.) and testosterone pellets (av. weight, 28.9 mg.; range, 28.4–29.2 mg.), and one capon served as a control and received a similar pellet of cholesterol. Counts were made every other day for 24 days and every fourth day thereafter.

The first increase in erythrocyte number was observed on the tenth day in both testosterone- and testosterone-plus-alpha-estradiol–treated capons. The red cells continued to increase, with the testosterone-plus-estrogen birds lagging slightly behind the others. The male level was reached in both groups on the twenty-second day. The doubly treated birds reached a peak of 3,830,000 erythrocytes per cubic millimeter on the thirty-second day, while the testosterone-treated capons reached a peak of 3,760,000 on the thirty-fourth day. In both groups the counts fluctuated between 3,000,000 and 3,400,000 for 112 days, when they began to fall to the control level. The capons with the double implants reached the control level on the 128th day, while those with testosterone pellets remained high until the 152d day. The estrogen-treated capons showed a slight downward trend but, in general, deviated little from the control (see Fig. 6).

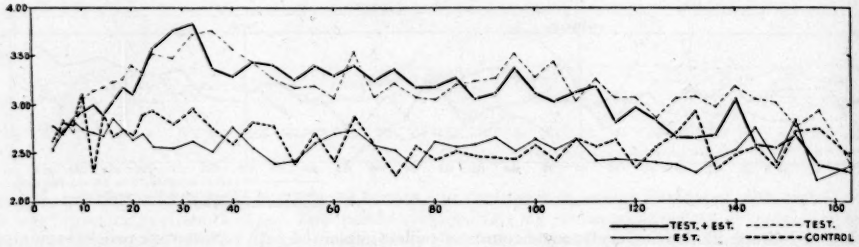

FIG. 6.—Graph illustrating erythrocyte counts of capons implanted with pellets of testosterone only, alpha-estradiol benzoate only, or both testosterone and estradiol. Testosterone pellets averaged 29.1 mg.; alpha-estradiol benzoate pellets, 27.6 mg. (*Ordinate*, erythrocytes in millions per cubic millimeter; *abscissa*, number of days after initiation of treatment.)

Although the estrogen absorbed from the pellets was adequate to produce feminization of the plumage in both the capons with estrogen pellets alone and in the double-pellet group, it would seem from these results that the amount of estrogen was insufficient for a blood-count effect, administered either alone or in combination with testosterone. These results seemingly contradict the observations on capons injected with alpha-estradiol, where a fall in the erythrocyte count was observed. However, in evaluating results, it must be borne in mind that the estrogen-injected birds received

a constant daily dose of 0.33 mg. for 25 days, whereas the capons with estrogen pellets had a total of 26.8 mg. available for the entire period from the time of implantation until complete absorption—approximately 130 days. Disregarding, for the moment, the gradually decreasing size of the pellets and assuming a constant rate of hormone absorption, this would amount to about 0.20 mg. per day. Actually, more than this would be absorbed during the early part of the pellet dynasty and less toward the end. The exact amount available at any time is not known, but conceivably it always was less than 0.33 mg. per day; and, if such were the case, the estrogen level presumably would be below the threshold for interference with erythropoiesis. Consequently, observations are being planned at the present time on capons which are to receive pellets containing twice the amount of estrogen used above.

It is interesting to compare the blood counts of the three capons which had testosterone administered in the form of pellets with those of capons which received testosterone injections (Fig. 1). The weights of the two groups were approximately the same. Each injected capon received a total of 72.0 mg. for 72 days, while each capon implanted with a pellet had only 29.3 mg. available for the entire pellet dynasty, which was approximately 130 days. The rate of comb growth, which is not indicated in Figure 6 because of the complexity of the graph, was similar in each group, although the combs of the injected capons were slightly larger. The first indications of comb regression came between the seventieth and the seventy-seventh days in both groups. For the first 36 days the rise in red-cell count was almost identical in the two groups; from the thirty-sixth to the eighty-eighth day the counts of injected birds averaged approximately 30,000 higher than those with pellets. After this time there was an abrupt drop in comb size and red-cell count in the injected birds, while the residual amount of testosterone in the capons with pellets may have been responsible for their slower and more gradual return to normal.

This would indicate that testosterone pellets of this weight are as effective as daily injections of 1.0 mg. of testosterone for approximately 36 days, after which they are seemingly less effective. This is probably due to the gradual decrease in the surface areas of the pellets exposed for absorption.

4. SEASONAL VARIATION IN ERYTHROCYTE NUMBER

Although the domestic fowl is not strictly a seasonal breeder, there is evidence for increased reproductive activity in the spring and early summer and decreased activity during the autumn and early winter, and these differences are correlated with changes in comb size. It seemed plausible to assume that such seasonal changes, if due to hormonal fluctuations, might also be reflected in seasonal changes in erythrocyte number. In the seasonal-breeding ground squirrel, a 37.8 per cent decrease in the number of red blood cells has been observed during the period of winter inactivity (Stuckey and Coco, 1942). Riddle and Braucher (1934) reported a seasonal difference in the red-cell counts of male and female pigeons and doves, with the highest counts in autumn and the lowest in summer. They observed no correlation between stages in the reproductive cycle and cell count.

If the fluctuation in hormone level is of sufficient magnitude to affect the number of red cells, one would expect the counts of roosters and sinistrally ovariectomized

poulards to be highest in the spring and lowest during autumn and early winter, while the lowest female counts would fall during the height of the laying season, when there is an increased estrogen production. On this assumption capons and bilaterally ovariectomized poulards should show no seasonal fluctuation attributable to sex hormones. Observations were made to test these assumptions.

Groups of roosters, hens, capons, and sinistrally and bilaterally ovariectomized poulards were observed at 3-month intervals for 24 months. Observations on the

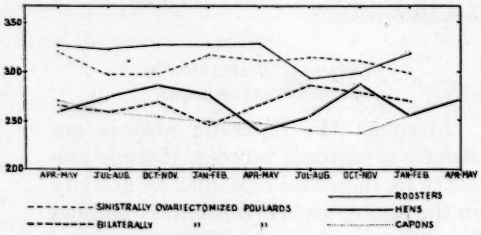

FIG. 7.—Graph illustrating seasonal erythrocyte counts. (*Ordinate*, erythrocytes in millions per cubic millimeter; *abscissa*, seasons.)

hens were continued for an additional 3 months. With the exception of the bilaterally ovariectomized poulards, where only ten birds were available, twelve birds were used in each group, and replacements were made whenever necessary to maintain this number. The birds were kept in indoor pens in which the temperature and light varied with the outdoor conditions in the late spring and summer. During the late fall, winter, and early spring the temperature was maintained at approximately 60° F., and, by means of artificial illumination, the period of light was kept constant at 14 hours. Figure 7 gives the average counts obtained for the five groups during the observation period.

With the exception of the females, which showed a definite tendency toward having their lowest averages in the spring and highest in the fall, there appeared no consistent trend in any of the groups. Aside from a drop in the counts made during the second summer and autumn, the number of erythrocytes varied little from season to season in the males. This was also true of the sinistrally ovariectomized poulards, suggesting, at least, that the level of male hormone is at all times sufficient to maintain a constant red-cell level.

Since these birds were kept under relatively constant conditions of light and temperature throughout the year, it seemed advisable to compare the winter counts of such hens and roosters with counts from birds which had been kept outdoors during the autumn and winter months, where they were exposed to the normally prevailing light and temperatures. Only three roosters and four hens were available for these observations. During the latter part of January, three erythrocyte counts were made, at 2-day intervals, on two lots of males and females, the one kept outdoors and the other in the poultry-house. These counts averaged 3,450,000 for the males and 2,960,000 for the females which had been kept outdoors, as compared with 3,180,000 and 2,540,000 for the roosters and hens kept in the poultry-house. The seven birds were then brought inside and exposed to the increased light and temperature conditions of the poultry-house. Four series of three counts each were made at 2-day intervals on each bird every other week for a period of 7 weeks. The average of the male counts was 3,580,000, showing a slight rise over the previous counts, and the average of the female counts was 2,420,000, showing a decided fall. The number of birds was probably too small and the changes in

red-cell number of insufficient magnitude to permit definite conclusions. Also we have observed that counts of control and experimental birds, kept under identical conditions, frequently varied in the same direction at the same time, indicating that environmental factors may be of significance in bringing about variations in peripheral blood values. However, the possibility that the changes noted above might be brought about by a stimulation in hormone production due to the supplementary light available in the poultry-house cannot be overlooked.

Lower red-cell counts and hemoglobin concentration have been reported previously for the laying hen (Harmon, 1936; Kakara and Kawasima, 1939); and others have reported a correlation between cyclic ovarian activity and the ossification of the marrow cavities (Kyes and Potter, 1934; Pfeiffer and Gardner, 1938; Kirschbaum, Pfeiffer, Van Heuverswyn, and Gardner, 1939). Similar cyclic changes in red blood cells and hemoglobin have been reported in pregnant and lactating rats (Reich and Dunning, 1943). However, in man, there appears little evidence for cyclic changes in red-cell production during the menstrual cycle or during pregnancy (Reich and Green, 1932; Smith, 1935; Leverton and Roberts, 1936; Pitts and Packham, 1939), and some investigators deny the relation between anemia and laying activity in the domestic fowl (Schultze, Elvehjem, Hart, and Halpin, 1936).

To investigate this further, erythrocyte counts were made on a group of eight hens at 4–6-day intervals over a period of 12 months. By the use of trap nests, accurate laying records were obtained for these hens. The results (see Table 2) confirmed our earlier observations on the existence of a seasonal cycle in females, with the lowest average erythrocyte number (2,410,000 per cubic millimeter) coinciding with the period of highest laying activity and the highest average erythrocyte number (2,990,000) falling at the time of lowest activity. The probability that such a difference in counts would occur by chance is less than 0.00005 per cent.

The increased estrogen production during the peak of the egg-laying season may be responsible for the observed cycle in the red blood cells. However, along with the increased estrogen production, there is also an increase in androgen secretion. It may be that the relative amount of androgen is insufficient to produce any stimulating effect on the erythropoietic tissues. Further experiments, now contemplated, with mixed androgen and estrogen treatment should help to clarify these points.

TABLE 2

SEASONAL ERYTHROCYTE COUNTS IN HENS

Months	No. Hens Observed	No. Eggs per Hen	No. R.B.C. Counts Made	Av. No. R.B.C.	Standard Deviation
Sept.–Nov...	2	6	38	2.96	.368
Dec.–Feb....	5	18	51	2.58	.351
Mar.–May..	6	23	81	2.39	.331
June–Aug...	5	22	60	2.40	.342

5. DIURNAL VARIATION IN ERYTHROCYTE NUMBER

A diurnal rhythm has been reported for white blood cells in man, dogs, and rabbits (Sabin, Cunningham, Doan, and Kindwall, 1925; Shaw, 1927; Tschishikow, 1927; Zirm and Bauermeister, 1933; Swan, 1943), with most authors agreeing that there are two distinct peaks, uninfluenced by rest, sleep, or nourishment, occurring near midnight and noon. Shaw

(1927), working with dogs, observed differences in erythrocyte counts, with low points at 9:00–10:00 A.M. and peaks at 3:00 A.M. and 4:00 P.M. In humans, Sabin, Cunningham, Doan, and Kindwall (1925) made red-cell counts at 15-minute intervals between 9:00 A.M. and 4:30 P.M. and observed highest counts at 12:15 and 4:30, with lowest counts at 11:30. Kleitman (1939) reported no evidence for a regular diurnal erythrocyte cycle in man.

TABLE 3

DIURNAL ERYTHROCYTE COUNTS

Types of Birds Used	Noon		6:00 P.M.		Midnight		6:00 A.M.	
	Red Cells (Millions per Cubic Millimeter)							
	Av.	Stand. Dev.	Av.	Stand. Dev.	Av.	Stand. Dev.	Av.	Stand. Dev.
Males	3.21	.397	3.43	.567	3.57	.483	3.36	.521
Females	2.56	.278	2.57	.487	2.69	.409	2.64	.391
Capons	2.43	.479	2.37	.240	2.42	.254	2.45	.336
	Time of Lowest Count (Per Cent of Individuals)							
Males	37.5		33.3		6.3		22.9	
Females	22.9		33.3		14.6		29.2	
Capons	20.8		33.3		18.8		27.1	
	Time of Highest Count (Per Cent of Individuals)							
Males	0		22.9		43.8		33.3	
Females	12.5		16.6		37.5		33.3	
Capons	37.5		8.3		37.5		16.7	

To determine whether a rhythm existed in the domestic fowl, twenty-four males, twenty-four females, and twenty-four capons were observed at 6-hour intervals during 24-hour periods. Table 3 gives the average counts for each group at noon, 6:00 P.M., midnight, and 6:00 A.M. Highest counts for both males and females occurred at midnight, although in females the differences in count during the 24-hour period were slight and statistically of no significance. When individual variations were analyzed, it was found that 43.8 per cent of the males and 37.5 per cent of the females showed their highest counts at midnight, with 0 per cent and 12.5 per cent of each group, respectively, having their highest counts at noon. Of the males, 37.5 per cent and, of the females, 22.9 per cent showed their lowest counts at noon, with only 6.3 and 14.6 per cent, respectively, showing their lowest counts at midnight. In capons the differences in red-cell concentration were less than those observed for females, and the percentage of highest counts for individuals was equally divided between midnight and noon. These observations seem to suggest a tendency in the males, and possibly in the females, toward a

diurnal rhythm in red-cell concentration, with the high point around midnight and the low point near noon. Statistical analysis showed that the probability for a chance occurrence of the difference observed between the noon and midnight counts of the males was 0.52 per cent, indicating that the difference is significant.

Since no significant variation was observed in females and capons, one might be tempted to conclude that the apparent rhythm in the male was correlated with a rhythmic male-hormone production. There is no good evidence for such a cyclic hormone production in the domestic fowl, although such a cycle has been observed in spermatogenic activity (Riley, 1940). Furthermore, all available evidence indicates that, should such fluctuations in hormone level exist, the fluctuations would probably be of insufficient magnitude to affect erythropoiesis. Likewise, it is extremely unlikely that variations in hormone concentration would be reflected immediately in an increased red-cell count, since we have shown, by observations following injections and pellet implantations, that about 8 or 10 days elapse before an increased erythrocyte count is observed. Factors other than male hormone might cause a cyclic red-cell production, destruction, or release of stored erythrocytes into circulation. Also diurnal shifts in tissue and circulating fluids might result in the observed rhythm, although there is evidence from studies on humans (Kleitman, 1939) that there is an entrance of tissue fluids into the circulatory system with an increase in blood volume during sleep. Since the horizontal position assumed during sleep is a contributing factor, such results might not hold true for birds. It is difficult, however, to understand why such a diurnal cycle exists to a lesser degree in females and not at all in capons.

6. THE THYROID AND ERYTHROCYTE NUMBER

a) Effects of thyroidectomy.—Red-cell counts were made on seventeen completely and eleven incompletely thyroidectomized adult males and on twenty-three completely and nine incompletely thyroidectomized adult females. The completely thyroidectomized bird shows a loss of melanin and an increase in red pigment of the feathers, a decrease in barbules, a reduced feather-growth rate, body dwarfism, and a juvenile reproductive system (Blivaiss and Domm, 1942*a*, *b*, 1944). Incomplete thyroidectomies show the same characteristics but to a lesser degree.

The red-cell counts of both completely and incompletely operated males were significantly reduced, the probability of chance occurrence being less than 0.00005 and 0.001 per cent, respectively, while the counts of females showed little change (see Table 4). There was a greater degree of reduction in the red-cell numbers of the completely thyroidectomized males than in those which retained or regenerated small thyroid fragments, but the difference cannot be regarded as significant. In three males and one female the blood was opaque and milky. This condition, which was observed in no other birds, was probably due to an increase in cholesterol or other complex lipids in the blood, and in the male it was associated with an extremely low erythrocyte count (1,270,000; 1,930,000; 2,320,000 per cubic millimeter). However, the one female in which the condition was observed had an abnormally high count (3,530,000). An increase in the cholesterol content of the blood has been observed in hypothyroid rabbits (Kunde, 1926).

b) *Effects of thiouracil.*—Thiouracil treatment in the brown Leghorn produces plumage changes similar to those observed in thyroidectomized birds (Domm and Blivaiss, 1944), although the thyroid gland itself becomes hypertrophied, as revealed by weight increase. Erythrocyte counts were made at 7-day intervals on six males, six capons, and three females, which received, orally, gelatin capsules of 0.3–0.6 gm. of thiouracil powder daily. Details of the technique used in administering the drug are described in the section on "Materials and Methods."

In the males and females the red-cell counts began to decrease around the fortieth day and stayed well below the normal average for the duration of treatment. Individual counts frequently dropped below 2,000,000 red cells per cubic millimeter. At various intervals treatment was discontinued on one or more birds, usually resulting in a rise in red-cell number within 12 days (see Figs. 8 and 9).

Similar results were obtained from observations on the capons, the red-cell count showing a fall by the thirtieth day. On the 128th day a count of 3,110,000 was observed in one individual; but with this exception the red-cell level remained well below the untreated-capon average (see Fig. 10).

During the course of these observations, three males, one female, and three capons died. Two of the males died as a result of fowl pox vaccination on the sixty-fourth and seventy-seventh days, respectively, of medication. Their red-cell counts a few days prior to death were 4,490,000 and 3,090,000 per cubic millimeter. In the former case the erythrocyte number had remained at the normal male

TABLE 4

ERYTHROCYTE COUNTS IN THYROIDECTOMIZED BIRDS

Types of Birds Used	Males			Females		
	No. of Birds	Av. R.B.C. Count	Standard Deviation	No. of Birds	Av. R.B.C. Count	Standard Deviation
Complete thyroidectomy	17	2.43	.663	23	2.60	.403
Incomplete thyroidectomy	11	2.62	.423	9	2.65	.262
Normal individuals	82	3.25	.434	84	2.61	.384

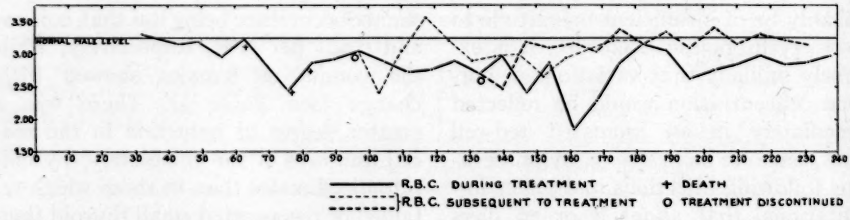
NORMAL MALE LEVEL
3.50
3.00
2.50
2.00
1.50
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240
R.B.C. DURING TREATMENT
R.B.C. SUBSEQUENT TO TREATMENT
+ DEATH
O TREATMENT DISCONTINUED

FIG. 8.—Graph illustrating erythrocyte counts of males treated with 0.3–0.6 gm. of thiouracil daily. (*Ordinate*, erythrocytes in millions per cubic millimeter; *abscissa*, number of days after initiation of treatment.)

level during the period of treatment; but in the latter bird the red-cell count had previously fallen to 2,180,000 and had remained low until the final observation before death. The third male and two capons died on the eighty-second, eighty-sixth, and ninety-ninth days, apparently as a result of the treatment. With the exception of the last count (4,510,000) tinued, remaining low (1,270,000 and 1,740,000) at the time of death.

DISCUSSION

The existence of a sex difference in the number of circulating erythrocytes has been well established for many species of animals, among which are rats (Adams and Shevket, 1929; Reich and Dunning,

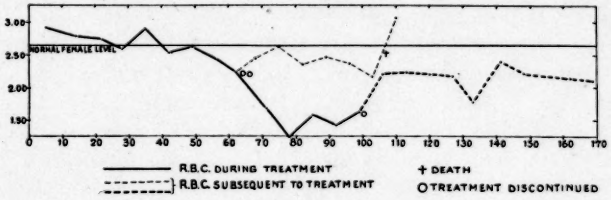

FIG. 9.—Graph illustrating erythrocyte counts of females treated with 0.3 –0.6 gm. of thiouracil daily. (*Ordinate*, erythrocytes in millions per cubic millimeter; *abscissa*, number of days after initiation of treatment.)

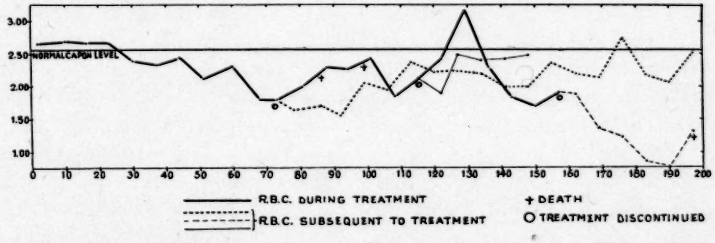

FIG. 10.—Graph illustrating erythrocyte counts of capons treated with 0.3–0.6 gm. of thiouracil daily. (*Ordinate*, erythrocytes in millions per cubic millimeter; *abscissa*, number of days after initiation of treatment.)

made on the rooster, the erythrocyte counts of both rooster and capons had been extremely low prior to death (2,170,000, 1,040,000, and 1,700,000). The cause of the sudden increase in red cells observed in the three roosters shortly before death has not been determined, though it may have been due to terminal hemoconcentration. The third capon and the hen died 40 and 39 days after cessation of medication. Their red-cell counts had been low during the period of medication and failed to show any signs of recovery after the treatment was discon-

1943), mice (Kamenoff, 1937), rabbits (Rosahn, Pearce, and Hu, 1934), dogs (Otto, 1885), cats (Lewis, 1941), sheep and horses (Storch, 1901), man (Otto, 1885), pigeons and ring doves (Riddle and Braucher, 1934), and the domestic fowl (Blacher, 1926; Chaudhuri, 1926; Forkner, 1929; Juhn and Domm, 1930; Landauer and David, 1933; Taber, Davis, and Domm, 1941, 1943; Domm, Taber, and Davis, 1943). On the other hand, observations made on golden hamsters (Stewart, Florio, and Mugrage, 1944) showed no sex difference of sig-

nificance. Böhmer (1939) reported no difference in the red-cell counts of men and women with similar constitutions living under similar conditions and concluded that the lower counts frequently found in females were not inherent but were merely due to a weaker constitution and a less active life.

In spite of some evidence to the contrary, it seems safe to conclude that, in general, a real sex difference in the red-blood-cell count exists in many animals. That this difference is mediated in part, at least, through the reproductive glands is suggested by results obtained from observations made on gonadectomized animals. Castration in the male rat, rabbit, or cock is followed by a drop in the erythrocyte count or in hemoglobin concentration, or both, while ovariectomy is followed by a rise or no significant change in count (Blacher, 1926; Ferrari, 1930; Juhn and Domm, 1930; Harmon, 1936; Steinglass, Gordon, and Charipper, 1941; Taber, Davis, and Domm, 1941, 1943; Domm, Taber, and Davis, 1943).

We have previously shown (Taber, Davis, and Domm, 1943) that sinistrally ovariectomized poulards have a higher red-cell count than unoperated females. We interpreted this difference to be due to androgens produced by the ovotestis, which develops from the right rudimentary gonad in such individuals. This supposition is supported by observations that testicular grafts (Juhn and Domm, 1930) or injections of male sex hormones (McCullagh and Jones, 1941; Steinglass, Gordon, and Charipper, 1941; Tyslowitz and Hartman, 1941; Vollmer and Gordon, 1941; Glass, 1943; Taber, Davis, and Domm, 1943) are effective in elevating the counts of male and female birds, rats, monkeys, or humans, while estrogen treatment induces anemia in chickens, rats, dogs, and monkeys (Castrodale, Bierbaum, Helwig, and MacBryde, 1941; Crafts, 1941; Steinglass, Gordon, and Charipper, 1941; Tyslowitz and Dingemanse, 1941; Tyslowitz and Hartman, 1941; Vollmer and Gordon, 1941; Von Haam, Hammel, Rorden, and Shoene, 1941; Taber, Davis, and Domm, 1943).

Our observations contribute additional evidence for the stimulating effects of testosterone on the erythrocyte number. We have also shown that it is not a temporary effect but one that may be maintained for an indefinite period by repeated pellet implantations.

In humans, following blood donations, hemoglobin and red-cell regeneration is slower in women than in men (Giffin and Haines, 1923; Fowler and Barer, 1942; Martin and Myers, 1934). Giffin and Haines believed that this was due not so much to hormonal factors as to the lower total mass of bone marrow in women, together with the fact that their habits, work, and diet are not so conducive to blood-cell regeneration as those of men. However, Finkelstein, Gordon, and Charipper (1944) induced anemia in rats by bleeding and found that males regenerated red cells more rapidly than females and that injections of male sex hormone stimulated regeneration in bled, unoperated and castrated males and females, while estrogen injections actually inhibited red-cell regeneration.

The rise in the number of erythrocytes after androgen treatment might be due to a stimulation of erythropoiesis or to a decrease in the rate of red-cell destruction. Conversely, the lower count in estrogen-treated animals might be due to an interference with erythropoiesis or to an increased rate of destruction. Observations on the bone marrow of estrogen-treated mice (Wentworth, Smith, and Gardner, 1940), rats (Gardner and Pfeif-

fer, 1938, 1943; Vollmer and Gordon, 1941), sparrows (Pfeiffer, Kirschbaum, and Gardner, 1940), pigeons (Pfeiffer and Gardner, 1938; Bloom, Bloom, Domm, and McLean, 1940), mallards and Pekin drakes (Landauer, Pfeiffer, Gardner, and Shaw, 1941), and the domestic fowl (Bloom and Domm, 1941) seem to support the former view. In these animals estrogen treatment stimulated the formation of endosteal bone in the marrow cavities of long bones. Correlated with calcification, hypoplasia of hemogenic tissues and decreased red-cell and hemoglobin concentration have been observed in rats. Testosterone treatment had no effect on the bone marrow or produced hyperplasia and increased erythrogenic activity (Steinglass, Gordon, and Charipper, 1941; Finkelstein, Gordon, and Charipper, 1944). Riddle, Rauch, and Smith (1944) likewise observed interference with erythrogenic tissues as a result of estrogen treatment in the pigeon, with most large blood vessels disappearing from the marrow area.

On the other hand, in spite of extensive marrow ossification, Bloom, McLean, and Bloom (1942) observed that erythropoiesis was not greatly affected in pigeons, and Jacobson (1944) reported no significant decrease in red blood cells in estrogenized mice and no interference with erythropoietic tissues. Although a drop in red-cell count was observed in rats, Von Haam, Hammel, Rorden, and Shoene (1941) likewise found no depression of hematogenic tissue in the bone marrow.

Of interest in relation to our observations on intersexual birds and birds treated simultaneously with androgen and estrogen is the work of Tyslowitz and Dingemanse (1941), who produced anemia in dogs by injecting androgens and estrogens together in 1:1 and 2:1 proportions. Gardner and Pfeiffer (1938) found only a slight increase in endosteal bone in rats after simultaneous treatment with alpha-estradiol benzoate and testosterone propionate over an extended period of time, while the same amount of estrogen alone produced extensive hypercalcification of femurs, compact and cancellous bone largely replacing the marrow cavities. They also observed that ovarian grafts into castrate males were more effective in producing bone changes than were similar grafts in normal males. Likewise, in estrogen-treated males bone changes are slower than in estrogen-treated females. Wentworth, Smith, and Gardner (1940) have shown that estrogen injections into mice increased the ash content of femurs and pelvic bones, while simultaneous treatment with androgens reduced or prevented this increase. These reports are in line with our observations on the simultaneous injection of androgens and estrogens into capons, where the degree of stimulation or inhibition of the erythrocyte concentration seemed to depend on the relative proportions of male and female sex hormones. The absence of any modifying effect of estrogen, when estrogen and androgen were administered simultaneously in pellet form, is not surprising, since capons with estrogen pellets alone showed no reduction in red-cell count, indicating that the amount of estrogen available was not sufficient to suppress erythropoiesis.

On the other hand, Bloom, McLean, and Bloom (1942) have reported a synergistic effect on the production of new medullary bone when estrogen and androgen were injected simultaneously into normal and castrate male pigeons during the fall or winter. This synergism has not been observed in other birds, such as the duck, sparrow, or domestic fowl; and

Riddle, Rauch, and Smith (1945) were able to obtain similar results in pigeons without the aid of androgen.

In view of this work on the relationship of the sex hormones to the number of circulating erythrocytes and to bone-marrow changes, it seems safe to conclude that the sex glands play an important role in controlling the number of erythrocytes, probably by influencing their production.

That the reproductive glands are not the only endocrine organs which have an effect on the concentration of circulating red cells has been shown by work on the thyroid, pituitary, and adrenal cortex.

Thyroid insufficiency has been reported to bring about a drastic reduction in erythrocytes and hemoglobin in rabbits (Kunde, 1926; Kunde, Green, and Burns, 1932) and in dogs, cats, and apes (Kishi, 1904); to interfere with hematopoietic reactions to reduced oxygen in dogs and rabbits (Mansfeld, 1913); and to delay blood regeneration after hemorrhage in rabbits (Furuya, 1924). Kunde has shown by blood-volume studies that this effect is not produced by blood dilution. Our observations indicate that thyroid insufficiency, as a result of thyroidectomy or thiouracil medication, produces a pronounced anemia in the fowl. Furthermore, our results suggest that the degree of anemia produced by thyroid deficiency may be found to be related to the degree of thyroid deficiency, since red-cell counts of incompletely thyroidectomized roosters were not so low as those of birds with no thyroid tissue.

Feeding or injecting thyroid substance brings about a rise in the number of erythrocytes in human anemic hypothyroids (Zondek, 1922; Hoskins and Sleeper, 1929), in thyroidectomized dogs and rabbits (Mansfeld, 1913), and in hypophysectomized rats (Vollmer, Gardner, and Charipper, 1941). Adams and Shevket (1929) first observed a rapid drop in red blood cells in thyroid-fed rats, followed by a rise to normal, suggesting that the thyroid hormone stimulated the bone marrow to increased erythrogenic activity and, at the same time, stimulated the spleen to increased red-cell destruction. The subsequent rise in cell number was possibly due to a later inhibition of splenic activity. Holler (1923) and Kunde, Green, and Burns (1932) reported an initial stimulation in rabbits, followed by a secondary anemia probably caused by a toxic-stimulating activity exerted by the thyroid. The opinion of the majority of investigators, however, is that the thyroid has no direct effect upon erythropoiesis but produces its effects indirectly by controlling the general metabolism and thereby creating an oxygen deficiency in hyperthyroid conditions.

Dougherty and White (1944) have presented evidence which suggests that the regulation of the number of red blood cells is under pituitary control and is mediated through the adrenal cortex. Adrenal insufficiency has been reported to cause a spectacular polycythemia and increased hemoglobin concentration in cats (Coney and Britton, 1932; Lewis, 1941) and in dogs (Rogoff and Stewart, 1926). Treatment with adrenal cortical extracts reduces the number of red cells in a few hours. This is believed to be due to changes in plasma volume and not to any actual increase or decrease in the number of erythrocytes.

Following hypophysectomy or in cases of insufficient pituitary secretion, there is hypoplasia of the bone marrow and a fall in the number of red cells in rats, dogs, and man (Houssay, Royer, and Orids, 1931; Meyer, Stewart, Thewlis, and Rusch, 1937; Flaks, Himmel, and

Zotnik, 1938; Overbeek and Querido, 1938; Querido and Overbeek, 1938; Vollmer, Gordon, Levenstein, and Charipper, 1939; Meyer, Thewlis, and Rusch, 1940; Vollmer and Gordon, 1941; Vollmer, Gordon, and Charipper, 1942); and in rats the normal erythrogenic response to low oxygen stimulation is not obtained (Meyer, Stewart, Thewlis, and Rusch, 1937). Meyer, Thewlis, and Rusch (1940) believed this to be due to a general metabolic effect rather than to loss of a specific hematopoietic hormone. Querido and Overbeek (1938), however, found that injections of posterior lobe extract or of thyrotropic, gonadotropic, or growth hormones failed to restore the red-cell level in hypophysectomized rats, while whole pituitary extract did; and Flaks, Himmel, and Zotnik (1938), using a deproteinized extract of anterior lobe, free from gonadotropic and thyrotropic hormones, produced an increase in erythrocyte concentration. They postulated another, less-well-known pituitary hormone, which might act by facilitating blood destruction, thereby indirectly stimulating the production of more red cells.

On the other hand, Meyer, Stewart, Thewlis, and Rusch (1937) reported an increase in reticulocytes, erythrocytes, and hemoglobin in hypophysectomized rats injected with growth hormone; and others have obtained stimulating effects with gonadotropic hormones from pregnant-mare serum and pregnancy urine (Goldman and Malavazos, 1941; Vollmer, Gordon, and Charipper, 1942). Hubble (1933), in a review article, summarized the evidence for pituitary control of the number of erythrocytes as follows: "Basophil cells of the anterior lobe of the pituitary stimulate all types of [blood] cells, giving rise to the clinical picture of polycythaemia. It is possible that part of this effect is produced indirectly through the thyroid and adrenal cortex." No mention is made of the gonads; but, in view of more recent work on sex hormones and the results obtained from pregnant-mare serum and pregnancy-urine therapy, it would seem safe to say that the lack of sex-hormone secretion in hypophysectomized animals may be a contributing factor to the subsequent marrow hypoplasia and change in red-cell ceunt.

SUMMARY

1. Earlier observations on the erythrocyte numbers of normal and gonadectomized chickens were confirmed. Averages were as follows: males, 3,250,000 red cells per cubic millimeter; females, 2,610,000; sinistrally ovariectomized poulards, 3,000,000; bilaterally ovariectomized poulards, 2,600,000; capons, 2,480,000.

2. Capons, injected daily with 1.0 mg. testosterone propionate, showed an increase in the number of red cells on the tenth day; the male level was attained on the eighteenth day and was maintained for the duration of injections (52 days) and for 16 days thereafter. The control level was reached 28 days after the cessation of injections. The rise and fall of erythrocytes were directly correlated with growth and regression of the comb.

3. Five-months-old pullets, implanted with pellets of testosterone propionate (av. weight, 44.2 mg.), showed an increase in the number of erythrocytes on the tenth day following implantation, at which time the male level was reached. This was maintained for 26 days. Counts returned to the control level by the fifty-fourth day. Similar cycles were observed following eight subsequent implants through a total of 690 days. Little or no comb regression was observed accom-

panying the fall in erythrocyte concentration toward the end of each implantation period, when pellets were small and almost completely absorbed.

4. One-month-old pullets, implanted with pellets of testosterone propionate (av. weight, 14.3 mg.), showed no change in red-cell count for 109 days. Subsequent implants (av. weight, 29.0 mg.) were followed by the characteristic rise and fall in erythrocytes, as described above. The lack of response in the younger pullets was probably due to the inability of the immature birds to respond or to insufficient hormone absorption from the relatively small pellets.

5. Three-months-old pullets, implanted with pellets of testosterone propionate (av. weight, 26.8 mg.), showed a slight rise in erythrocyte count. Birds receiving two pellets on the 80th and 180th days had red-cell counts higher than those receiving only one, suggesting that the rise in erythrocyte number is, within limits, proportional to the amount of hormone present. This would seem to indicate that the results obtained in 1-month-old pullets were due in part to insufficient hormone concentration. Other evidence indicates that the age of the bird also plays an important role.

6. Capons, injected with 0.33 mg. alpha-estradiol benzoate plus 1.0 mg. testosterone, showed a rise in the red-cell count paralleling testosterone-injected controls.

Capons, similarly injected with 0.75 mg. alpha-estradiol plus 1.0 mg. testosterone, showed an early decrease in the red-cell number, followed by a subsequent rise to the normal level.

Capons injected with 0.33 mg. alpha-estradiol alone showed a decrease in the red-cell number.

This suggests that small amounts of estrogen have neither an inhibitory nor a synergistic effect when combined with androgens, while larger amounts of estrogen tend to counteract the effect of androgens.

7. Capons implanted with testosterone and alpha-estradiol pellets of approximately equal weights showed a rise in the red-cell count paralleling controls implanted with testosterone alone, while estrogen pellets alone had no effect on the red-cell count.

8. Hens showed significantly lower counts in winter and spring and higher counts in the autumn, but no seasonal trend was observed in males, capons, or poulards. Lowest blood counts in hens coincided with the period of greatest egg production, suggesting that the increased production of estrogens at that time, in spite of the simultaneously increased androgen, may be a factor in producing the low red-cell count.

9. A significant diurnal variation in the number of erythrocytes in the blood, with the highest point at midnight and the lowest point at noon, was observed in roosters. In hens the degree of variation was much smaller than in the males, but the same general trend was observed. Capons showed no consistent rhythm.

10. Thyroidectomy resulted in a significant fall in the number of erythrocytes in males but no noticeable change in their number in females. In cases of incomplete thyroidectomy, the reduction in erythrocytes was not so great.

11. Thiouracil treatment lowered the red-cell counts in males, females, and capons. These counts frequently fell below 2,000,000 red cells per cubic millimeter.

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A STUDY OF SOME EFFECTS OF SEX HORMONES UPON THE EMBRYONIC REPRODUCTIVE SYSTEM OF THE WHITE PEKIN DUCK[1]

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I. INTRODUCTION

Since Lillie (1917) made the statement, at the conclusion of his now famous classic on the freemartin, that sex differentiation was controllable within variable limits in certain groups and that to determine the means, limits, and subsequent results of such control was one of the most important tasks of biology, many investigators have delved into the mysteries of experimental sex control. Dr. Lillie asserts in this paper:

> In the case of the free-martin we do not find that hormones cause the development of any structure which is not represented embryologically in the normal female; the hormones act in this case merely by inhibition or stimulation of normal embryonic rudiments. If this should hold as a principle also on the male side we could not expect that the transformation of a testis into an ovary should ever occur, although the suppression of complete testicular development would probably happen.

In all the years that have elapsed since this investigation was made, very few exceptions have been found to the first part of the above statement, namely, that hormones cannot cause the development of any structure not embryologically present in the normal female; however, in several classes of vertebrates, a biological basis for a transformation in the male to the female direction has been found. Risley (1933) in the class Reptilia found that in the male musk turtle the gonads had both cortical and medullary components, demonstrating potential development in the female direction. Risley also has observed (unpublished data) that the alligator testis attains morphological sex differentiation without the complete loss of cortex (Forbes, 1938, p. 350). Among birds, Laulanié (1886) recorded the presence of a potential cortex on the developing left testis in the male chick, which was regarded by Swift (1916) as of historic interest only; hence it passed into obscurity until Burwell (1931) observed such a potential cortex in the duck. Other birds are known to have this structure also; among them are the sparrow (Witschi, 1935) and the robin and the tern (Willier, 1939). In several mammals—the cat, dog, squirrel, guinea pig, mouse, rat, pig, sheep, calf, and man—Hett (1930, 1932) records the presence of a persisting germinal epithelium on the male gonads. This recital by no means exhausts the list and has not taken into consideration the status of the Anamniotes, wherein there are certain forms, such as *Bufo vulgaris* and *Rana temporaria* (Witschi, 1929) and others, that undergo a spontaneous sex reversal, indicating that these individuals are potentially bisexual.

With the discovery of a potential testicular cortex in the males of these various groups and the isolation and increasing availability of sex hormones in pure form, efforts to duplicate the "freemartin effect" were intensified, the use of hormone preparations largely replacing the older grafting experiments.

It was hoped that the effect of hormones upon the persisting germinal epithelium observed on the left testis of the duck embryo (Burwell, 1931) could be ascertained immediately, but uncontrollable circumstances delayed this work for a number of years. Meanwhile, it was established for the chick that this thickened germinal epithelium could be induced by homologous hormones to develop into cortex which brought about an intersexual condition or even complete inversion of the left testis (Kozelka and Gallagher, 1934; Wolff and Ginglinger, 1935*a*; Dantchakoff, 1936; Willier, Gallagher, and Koch, 1937).

In view of these important findings in the chick, it was decided to determine to what extent the response of the urino-

genital system of the duck to hormone treatment corresponds to the behavior of that system in the chick. The purpose of this investigation, therefore, was to ascertain (1) the normal behavior of the persisting germinal epithelium and the period of its duration in the duck; (2) whether complete inversion of the testes is possible by treatment with estrogens; (3) how late in the incubation period the germinal epithelium is modifiable by hormones; and (4) the effect of androgens upon the urinogenital system.

II. MATERIALS AND METHODS

The White Pekin duck was used throughout this investigation. Eggs were obtained from a farm in Indiana and a hatchery in Ohio.[2] The majority of these were shipped via express in cartons designed for duck-egg transportation and, with the exception of one lot, showed no evidence of improper handling in transit. As a rule, the eggs were unpacked and placed on trays in a cool basement, where they were allowed to stand for 24 hours, or overnight, before being placed in the incubator; however, toward the close of the experiments we were informed[3] that this practice was no longer followed by many hatcheries, and thereafter the eggs were usually placed in the incubator upon arrival.

The incubators were electrically heated, thermostatically controlled, and provided with moisture by means of a sand tray. Moisture was not added prior to the 4th day of incubation, in order to permit a certain amount of evaporation and thereby obviate the danger of hormone's oozing through the perforation when the eggs were injected. Only rarely did any exudation of fluid occur when this procedure was followed. When exudation did occur, the egg was slightly moved from side to side for a few seconds, during which time the exudate was usually withdrawn into the opening. From the 4th to the 15th day the sand was kept fairly moist, and the eggs were sprinkled daily after the 10th day with water heated to 37°–39° C., as suggested by Rolnik (1943). After the 15th day the humidity was increased by adding additional water, and during the hatching period an even greater amount of water was added.

The temperature was kept between 101° and 102° F., and an attempt was made to subject the eggs to a fluctuating temperature, such as they undergo under natural conditions. This was accomplished by cooling the eggs at least twice a day after the first 24 hours for 5-minute periods, and after the 15th day the cooling period was prolonged to approximately 10 minutes. The eggs were turned at each cooling period.

In order to avoid waste of hormone, injections were not made until fertility was determined. Candlings were subsequently made at regular intervals in order to remove dead embryos.

For the injections, instruments designed and previously used by Dr. Domm were employed, and methods devised by him in his experiments on chicks were used. A 1-cc. hypodermic syringe with needle shortened to a length of approximately 3.0 mm. was employed. To puncture the shell and underlying membranes a pin vise, with inserted pin similarly shortened, was used. This precaution was taken in order to prevent injury to the embryo and surrounding vessels. All instruments were washed and sterilized immediately preceding and following injections and were kept between the

[2] Mrs. Blanche Tormohlen, Portland, Indiana, and the Twentieth Century Hatchery, New Washington, Ohio.

[3] By Dr. H. J. Sloan, Department of Agriculture, Poultry Section, University of Minnesota.

folds of a clean towel while the eggs were being handled. Each egg was candled just before injection, to determine the location of the embryo, and a spot was marked on the shell for the aperture in close proximity to the embryo, care being exercised to avoid the large extra-embryonic blood vessels. The site of injection on the shell was sterilized by wiping with a small wad of cotton moistened in 70 per cent alcohol. The egg was placed in a small cardboard box containing cotton to hold it in position while it was being injected. The hormone was always deposited within a radius of about 0.5 cm. from the embryo. Injection age of embryos ranged from 3 to 18 days' incubation. (Table 2, 1st col.). The small puncture was sealed by applying melted rubber paraffin.

A $\frac{1}{20}$- or $\frac{1}{10}$-cc. solution of hormone of varying concentration was injected at a time, and the egg was returned to the incubator with the sealed aperture turned slightly from the topmost position so that the embryo would remain close to the introduced hormone but, at the same time, not directly under the sealed puncture. This procedure reduced adhesions and consequent death of the embryos. Some of the embryos received a second and some a third injection on subsequent days (see Table 2).

The time of killing of the experimental birds ranged from 10 days' incubation to 24 days' post-hatching. All of these were examined under a binocular dissecting microscope to note and record any changes in the gonads or ducts, and many gonads and some ducts were sectioned for evidence of histological change (see Tables 3, 4, 7, and 8).

Sesame oil, the hormone solvent, was injected into 20 control eggs in the same manner and amount as the hormone solutions. The other controls received the same manipulation as experimentals but were allowed to develop untreated.

During the experimental period, a total of 2,793 eggs was handled. Treatment accorded these is shown in Table 1.

In the course of the investigation three estrogens and one androgen were employed. The estrogens were alpha-estradiol benzoate (Progynon-B), diethylstilbestrol, and hexestrol. The androgen used was testosterone propionate.

TABLE 1

SUMMARY OF DATA ON TREATMENT OF EGGS

Dead or Infertile	Untreated	Sesame Oil	Progynon	Hexestrol	Stilbestrol	Testosterone	Total
806	389	20	928	540	45	65	2,793

III. NORMAL DEVELOPMENT

In an earlier study (Burwell, 1931) the development of the duck gonad was traced from its incipiency as germinal epithelium through the 9th day, when sex differentiation had occurred. Up to that time the accessory ducts showed little or no differentiation. In the present investigation the condition of the reproductive system was studied, both macroscopically and microscopically, from the 10th day of incubation through the 24th day after hatching.

A. DEVELOPMENT OF THE OVARIES

Directing attention now to the gonads, we find that those of the female present the asymmetry characteristic of

most birds (Domm, 1927, and others). A macroscopic inspection revealed some variation in size of the right rudimentary gonad in duck embryos of the same age; yet in all cases examined it was readily observed, and in some instances the rudiment was quite prominent.

Many of the female gonads of the normal series were not prepared for microscopic study, but a fair sampling of from 10 to 26 days of incubation was studied to obtain an idea of the normal condition for comparison with experimental individuals.

1. *Left ovary.*—At 9 days' incubation, shortly after the time of sex differentiation, the germinal epithelium on the left ovary consists of several layers of cells containing scattered primordial germ cells with an underlying compact medullary tissue (Burwell, 1931). The picture has changed considerably by the 10th day. A decided line of demarcation, filled with a filmy network of connective tissue, has appeared between the epithelium and the medulla; and a sharp increase in the number of primordial germ cells has occurred, with definite beginnings of cortical cords, which cause this layer to be thicker than on the previous day. There seem to be very few primordial germ cells in the medullary portion of the left ovary, in contrast to the condition found in the right.

On the 12th day the cortex on the left ovary has increased in thickness and is composed of numerous cords. The germ cells have decreased somewhat in size and the area of filmy tissue between cortex and medulla is not so wide in extent. In the 14-day stage the left ovarian cortex is more compact in appearance, and the cells composing it are very uniform in character, instead of being grouped as definitely in cords as in the previous stage. The thinned-out area between cortex and medulla is reduced in width; also very occasionally a group of differentiated cells, characteristic of the fully differentiated cortex, is found. These cells, which have been used as a criterion for differentiated cortex, are recognized by their large, deeply staining nuclei, with their compact mass of chromatin near the center of the cells surrounded by a clear area of cytoplasm, giving the cortex a much lighter appearance than the rest of the gonad (see Fig. 17). These differentiated cells are in the stage of synizesis.

At 18 days the patches of differentiated cells in the cortex of the left ovary have increased in number, so that the entire cortex, with the exception of the lateral margins and the anterior and posterior tips, has been transformed. By the 22d day, differentiation of cells in the cortical layer has extended to practically the anterior and posterior tips of the left ovary; but the lateral margins still show a border of undifferentiated cells on either side of the point of attachment, as seen in cross-section. This condition in the left ovary of a 22-day embryo is illustrated in Figure 16. On the 26th day the undifferentiated cell area mentioned for the 22d day usually has been transformed entirely into differentiated cells.

2. *Right rudimentary gonad.*—At 9 days' incubation the germinal epithelium on the right ovary is comprised of one or sometimes two layers, with numerous spaces between it and the underlying medulla. By the 10th day the right ovary is covered with a single layer of epithelium, which contains scattered primordial germ cells and in some cases a few large cortical cords that push out into the filmy area between the epithelium and the underlying medullary portion. Numerous germ cells, single or contained in cords, are found in the interior

of the gonad. Some germ cells lie very deep, so that the gonad in some cases is not separated into cortex and medulla as in the left one and only the single-layered epithelium stands out clearly from the rest.

On the 12th day the cells comprising the epithelium covering the right gonad have flattened, making this layer even thinner than on the 10th day. In some cases lumps of cortical tissue are found on the surface or, in others, scattered in the deeper portion of the ovary. At 14 days there is a heterogeneous grouping of lumps, which appear to be cortical in nature, and medullary tissue with occasional spaces which indicate degeneration. The lumps attached to the epithelium in some sections are reminiscent of the thickened condition of the epithelium found in sections of the left testis in untreated males of comparable age.

Although the right rudimentary gonad does not exhibit well-defined cortex and medulla at the 18th-day stage, there may be found among the cells which constitute its bulk some scattered cells resembling those in synizesis in the differentiated cortex of the left ovary. These are believed to be derived from the primordial germ cells, which were seen in the medullary tissue in earlier stages; and they form, in certain instances, cords that contain numerous cells—all in synizesis—that appear no different from cortical cords with cells in synizesis in the cortex. In some treated cases, even the medulla of the left ovary contains cords of these cells in synizesis that seem the same as those in its cortex. The bulk of the right ovary, therefore, in many instances displays a mixture of medulla and cords with cells that appear to be descended from the primordial germ cells and are comparable in a large measure to cortical cords. On the 22d day the right gonad may show, near the periphery, patches of cells in synizesis. One embryo of this age also shows a few cells of this appearance among the medullary cords, which are probably derived from the primordial germ cells.

A most interesting observation was made upon the right rudimentary gonad of one 26-day embryo. In addition to patches of cells in synizesis among the medullary cords, there was noted in the deepest portion of the gonad a few tubules which were decidedly testicular in appearance. In none of the nine untreated females studied up through the 26th day did we find a well-defined cortex; yet, on the other hand, there was no individual which failed to show some cortical lumps, either very scattered or fairly numerous.

Since the chief interest in the female was to ascertain the normal appearance of the ovary throughout its various stages of differentiation, histological study of normal females was carried no further than the 26th day of incubation.

B. DEVELOPMENT OF THE TESTES

While it has been known for some time that the ovary possessed the structural basis for sex reversal in the medulla (Lillie, 1927; Domm, 1927, 1929; and others), it was generally believed that the testis in higher forms lacked the homologue of the ovarian cortex. Laulanié (1886) noted a thickened germinal epithelium on the left testis of the chick, which persisted from the 7th to the 11th days of incubation, and he regarded this as homologous to the cortical layer of the ovary. Kozelka and Gallagher (1934) injected male human urine extracts—theelin and theelol—into hen's eggs and caused the development of ovotestes on the left side in male embryos. These results, therefore, clearly indicated the ability of this *couche ovigène* of Laulanié

to develop into ovarian cortex, although these investigators did not so interpret their findings. Similar phenomena have since been demonstrated by a number of other investigators. Other instances in which this persisting epithelium has been noted on the testis are Burwell (1931) in the duck; Hett (1932) in the cat, pig, sheep, calf, man, dog, squirrel, guinea pig, mouse, and rat; Risley (1933) in the musk turtle, also the alligator; Witschi (1935) in the sparrow, and Willier (1939) in the robin and tern.

1. *Left testis.*—On the 9th day of incubation, soon after the time of sex differentiation, the left gonad of the male duck embryo shows a well-defined germinal epithelium, consisting of cells with rounded nuclei separated from the sexual cords by a basement membrane (Burwell, 1931). The epithelium of this gonad shows an occasional germ cell at this stage. These cells gradually increase in number, and on the 12th day a germ cell here and there becomes multinucleate, causing the appearance of lumps on the margin of the gonad. The number of nuclei, which do not always have well-defined cell walls, varies from two to eight or more. These lumps increase in number and in size through the 14th and 16th days and in some instances appear to fuse to form a continuous layer. Figure 18 shows a 14-day embryo with lumps of various sizes developing from germ cells in the epithelium, while Figure 19, of a 16-day embryo, illustrates apparent fusion of these lumps on the gonad to form a continuous layer.

A maximum thickness appears to be attained on the 24th day, and male embryos of 14, 16, 18, 20, 22, and 24-days exhibit a condition of the left gonad which is indicative of the fact that the germinal epithelium proliferates cords of cells that form a rather imposing layer. Figure 20 illustrates such a layer near the caudal end of a 24-day normal male, and Figure 21, from the same embryo, shows the nature of this layer about the midportion of the gonad. These new cords of cells differ from the old, in that the peritoneal nuclei have not arranged themselves on either side of the cords in a compact layer with germ cells toward the center, as the old cords have, but are scattered uniformly in a dense condition throughout the cords. The arrangement of the germ cells in the older cords is in agreement with the observations of Swift (1916), who noted that the germ cells were not confined to the center of the tubules in the chick. Semon (1887) and Popoff (1908) held that the germ cells in the chick were placed centrally within the tubules.

A total of seventeen male embryos, ranging in ages between 14 and 24 days, was examined, and all showed development of these cords in varying degrees, some slight but in most cases pronounced. There would seem to be no doubt that the epithelium is proliferating a second set of cords.

Grünwald (1934*b*) describes the development of secondary sexual cords, *Keimstränge*, in the human testis, which are analogous to the secondary sexual cords of the female. To his knowledge these had not been observed before. Although Hett (1930) records the presence of cords of testis epithelium, he does not trace their development into typical sexual cords. Grünwald did not see these cords in all embryos studied, and he had not determined at the time why they were sometimes absent; when they were noted, they were found on both testes. He observed that, as development proceeds, these cords make numerous unions with the primary cords and later cannot be distinguished from them.

In the case of the duck it appears that the large secondary cords become incorporated into the testis and lie intimately alongside the primary cords. Figure 19 shows the left testis of a 16-day untreated male embryo, which is suggestive of such a fate. This illustration is from an embryo 2 days older than that in Figure 18, which illustrates an earlier stage in the formation of the secondary cords. By continued development and more or less fusion, the result is a fairly continuous layer in apposition to the primary cords, as revealed in Figure 19. In the thirteen other embryos examined, with ages ranging from 16 through 24 days, at which time maximum thickness is reached, there was indication of varying degrees of fusion of cords to form an outermost layer. This layer in these cases might be composed entirely of separate cords, partially fused cords, or nearly completely fused cords.

Observation of actual connection between primary and secondary cords, as recorded by Grünwald for man, has not been noted in the duck, and it is not certain what the fate of these secondary cords is; but a 28-day-old embryo which shows a group of these newly formed cords inside the tunica albuginea, in close contact with the primary cords, is suggestive of a similar destiny (Fig. 22). After the 24th day of incubation the investing outer layer of newly formed cords becomes less obvious as such, as revealed by three embryos, 26 days old. One of these showed hardly a trace. In addition to the 28-day-old embryo already mentioned, four others of that age were examined, and one of these showed a marked degree of fusion of secondary tubules in contact with the primary tubules near the posterior end of the gonad, one possessed a few small lumps at the posterior end, and two exhibited a very thin layer in places which contained some germ cells.

After hatching, the layer of secondary cords on the left gonad becomes more and more inconspicuous. This was shown by observations upon ducklings of 1, 2, 4, 6, and 8 days post-hatching. The latest age at which fairly large lumps were noticed in connection with the epithelium was 2 days. At 8 days, only an occasional flattened lump was found, which represents the remnants of the epithelium. In 12-, 16-, and 26-day-old post-hatch birds, these lumps became even more scarce, and the one male duck among the three killed on the 24th day showed no trace of them. Just a minute trace was found on only one of the two 20-day-old birds. It would seem from this that practically all traces of thickening in the epithelium vanish by the 24th day after hatching or shortly thereafter. Primordial germ cells in the secondary cords were noted last in a duck of 16 days' post-hatching.

2. *Right testis.*—At 9 days the cells composing the outer layer of the right testis are much flattened, by way of contrast to the rounded ones comprising the persisting germinal epithelium on the left testis, and are set off by an incipient tunica albuginea. As incubation proceeds, the area between the testicular cords and the epithelial layer widens, as a loosely organized connective tissue develops within it. By the 20th day the connective tissue has become more compact, containing many flattened nuclei; and on the 24th day these flattened nuclei have come into intimate contact with the outer epithelial layer, aiding in forming the tunica albuginea proper and leaving spaces intermittently between this structure and the testicular cords.

Once in a great while the right testis may display a feeble attempt to develop a germinal epithelium. This is manifested

in later stages by an occasional thickened lump, but in no instance was a well-developed germinal epithelium observed on the right testis, as was found in many instances on the left.

Multiplication of the tubules within the gonads produces on the surface of the gonad, in some instances, fine ridges or elevations. These are seen as early as 18 days' incubation and are not evident in all normal embryos. At the caudal end the proliferating tubules sometimes extend from the surface of both gonads onto the surface of the underlying mesonephros, producing small, flattened, finger-like processes, with the result that the gonad margin is not smooth and rounded but is diffuse in character.

By way of summary of testicular development, the persisting germinal epithelium covers the entire left gonad in a well-defined layer of more or less uniform thickness at 10 days' incubation. As development proceeds, this structure tends to become thinner and eventually disappears anteriorly while thickening posteriorly, the maximum amount of increase in depth being attained caudad of the mid-line of the gonad, where it appears that the layer may give rise to secondary sexual cords in some instances. There is a possibility that these secondary cords may be incorporated into the gonad along with the primary.

On the right gonad, vestiges of this layer are present from time to time; however, no continuous epithelium was found on this testis at any time. Since this epithelium is lacking to any marked degree, the reason is apparent why the right testis does not undergo inversion or partial inversion, as the left does when treated with estrogens. This gonad also apparently fails to proliferate secondary cords, as the left does normally.

C. MÜLLERIAN DUCTS IN THE MALE

The Müllerian ducts in the male appear to reach their maximum development about the 10th day, for in some 11-day males signs of degeneration are apparent. As was observed by Lillie (1919) in the case of the chick, retrogression begins posteriorly and proceeds anteriorly. There is a thinning of the thick-walled duct, which becomes more and more pronounced until a mere line indicates the original position, and then this line disappears. This process of degeneration extends from about the 11th to approximately the 18th day, when the vanishing-point has been attained. The extreme anterior tips have been noted to persist until as late as the 26th day. The period of retrogression appears to be of longer duration than that described by Lillie (*ibid.*) for the chick, in which it is stated that development is complete on the 8th day and degeneration is at an end or far advanced on the 11th day. Willier (1939) observes that disappearance is complete by the 18th day in typical cases.

D. MÜLLERIAN DUCTS IN THE FEMALE

In the female, degeneration of the right Müllerian duct proceeds in the reverse direction of that in the male, namely, from anterior to posterior end. In 12-day females the right duct is still entire and has the appearance of that of a 10-day male, whereas the left shows indications of differentiation into the definitive adult form. In females of 13 days, the anterior end of the Müllerian duct on the right side begins to recede, while differentiation on the left is more pronounced. By the 14th day, regression on the right has reached the posterior end of the Wolffian body, or approximately so. On the 16th day the anterior, or mid-level, of the shell gland has been attained, and

the 18th day reveals a shrunken vestigial tip adjoining the cloaca, which persists apparently indefinitely. The left duct has completed its differentiation at this stage. In neither male nor female did we observe an abnormal persistence of Müllerian ducts in normal individuals.

IV. EXPERIMENTAL

A. RESULTS OBTAINED WITH ALPHA-ESTRADIOL BENZOATE (PROGYNON-B)

In the use of this hormone there was a variability in the time of injection, the amount of hormone used, and the age at which the embryos were killed. These data, along with the number of eggs injected and survival percentages, are summarized in Table 2.

A total of 356 embryos was examined macroscopically to determine the effects of Progynon on the reproductive system. Of these, 181 were males and 175 females. The males were distinguished by less disparity in gonad size, macroscopically, and by the histological character of the primary cords. Of the 181 males, 124 were studied microscopically also. A glance at the survival percentage in Table 2 will reveal that the mortality rate is very high, especially just prior to hatching and late in the season. The dosages employed ranged from 0.25 to 1.5 mg. Because of the low degree of solubility of the hormone, 5 mg. per cubic centimeter, the quantity of material which would have had to be injected in order to increase the dosage precluded larger doses in the early stages. The largest dose, 1.5 mg., failed to bring about complete sex reversal of the testis, although a high degree of intersexuality was attained. Willier, Gallagher, and Koch (1937) found that 1.0 mg. of theelol was adequate to bring about complete reversal of the left testis of a zygotically determined male chick into an ovary; Wolff and Ginglinger (1936*a*) effected complete reversal of the left testis in the chick with 0.05 mg. of folliculin; and Dantchakoff (1936), by using 2,500 mouse units of folliculin, was able to produce the same result. These investigators, however, used different methods of administering the hormones, which, no doubt, accounts for the differences in amounts of hormone required to bring about complete reversal.

On the basis of these comparisons it would seem that complete inversion of the testis is not possible in the duck or that larger quantities of Progynon are requisite to bring about this change in the chick than are required of other estrogens.

In view of the absence of complete inversion and the failure of Progynon to effect such profound changes in the duck as those described by other investigators in the case of the chick, it seemed best to discuss results from the standpoint of dosage, time of injection, and degree of modification at the time the embryos were killed. A study of the normal stages of gonad development in the female reveals that, in the 14-day embryo, synizesis may be observed in a few cortical cords. As development proceeds, this condition may be noticed in more and more cords, so that, by the 18th day, only anterior and posterior tips and the margins bordering the hilus are not in synizesis. This condition has been used as a criterion for fully differentiated cortex.

The earliest stage in which fully differentiated cortex was observed on the left gonad of the male after treatment with Progynon was at 22 days following injection on the 5th day (Case 4P1522 ♂ b). However, cases have been noted in which cortical cords are definitely recognizable as early as 14 days after treatment, the embryo being 18 days old (Case 13P1418

a♂). The very youngest individual in point of injection age to show incipient cortical cords was 13 days after injection and at 18 days' incubation (Case 8P2518♂b). With these facts in mind, the cases discussed in Groups I, II, III, IV, and V include only birds in which a minimum of 14 days had elapsed since treatment.

in synizesis. One of these was injected on the 3d day, one on the 5th, and two on the 8th. The reproductive system of the 3-day injected case was somewhat more highly modified than the 5-day injected embryo, as is shown in Figure 3. Comparison with the normal male and female systems, Figures 1 and 2, respectively, reveals the intersexual condition ex-

TABLE 2

DATA ON THE EFFECT OF PROGYNON

Injection Age* (Days)	Quantity Injected (Mg.)	No. of Eggs Injected	Embryos Killed				Survival (Per-age)
			Age*	No.	Male	Female	
3	0.25	14	18–26	14	5	5	71
4	.....	30	22–26	21	6	10	53
5	.....	94	10–26	72	12	17	30
8	.....	33	22–26	21	8	8	48
3	0.50	18	26	6	2	0	11
4	.....	182	16–26	120	32	30	34
5	.....	90	14–26	78	14	7	23
6	.....	52	14–26	40	13	17	56
10	.....	54	16–8 †	54	17	11	51
12	.....	83	18–2 †	67	20	22	50
14	.....	48	20–8 †	49	16	17	62
16	.....	22	22–28	18	3	11	72
18	.....	34	22–27	30	11	8	52
4 and 6	0.75	28	20–26	14	4	5	32
4 and 6	1.00	31	20–26	15	9	2	35
4, 6, and 8	1.50	63‡	25	23‡	8	5	20
Totals of sexes	.....	.....	.....	.....	181	175	.....
Sex ratio (per cent)	.....	.....	.....	.....	51	49	.....

* Incubation days.

† Post-hatching.

‡ The discrepancy in all cases in the table between number of eggs injected and embryos killed is due to the death of embryos in the interim.

1. *Males: Group I.*—The dosage in this instance was 0.25 mg., and the ages at the time of injection ranged from 3 to 8 days. A total of twenty-one male embryos was examined macroscopically and microscopically. Of these, nine were 26 days old and so had the longest period in which the hormone might exercise its influence. Four of the nine 26-day embryos had cells in the cortex which were

hibited by this embryo, which was clearly visible to the naked eye. These three figures represent embryos of the same age and are drawn to the same scale. The gonads are clearly of an intermediate type. They are more flattened than the male and more rounded than the female. The right is approximately the size of the left, but all along its lateral margin are detached lumps of testicular tissue.

In the left gonad, microscopic study discloses a thin surface layer of cortex, with many cells in synizesis but not uniformly distributed, extending over most of the gonad surface (Fig. 24). It does not extend down to the hilus over the medial or lateral border, or to the anterior tip. The layer becomes thinner and confined more to the medial margin as the anterior end is approached and finally disappears rather close to the tip. Posteriorly, there is a gradual increase in thickness; but the thicker, even layer gives way to a lumpy cortex, which appears less differentiated in character. These lumps extend to the posterior end, and some of them are continuous with outgrowing primary cords. The cortex for the most part, however, is cut off from the underlying testicular cords, which in many instances push up against the cortex or among its cords. There is little or no difference in character in these secondary sexual cords, which occupy a cortical position, and the normal testicular cords of untreated males.

On the right gonad there is no definite cortex but only an occasional flattened patch of cells outside the tunica albuginea. Medullary cords are not apparent in either gonad.

The Wolffian ducts in no instance show effects of Progynon administration. The Müllerian ducts in the case just described show almost typical female development, the exception being a disparity in shell-gland size in favor of the normal.

One of the two embryos injected on the 8th day showed as good, if not better, differentiation of cortex as did the 3d-day injected embryo. The other exhibited a very thin layer of differentiated cortex on the posterior portion. In both cases the Müllerian ducts had developed into cystlike structures at the anterior ends, with no enlargement posteriorly. These cysts were filled with a watery fluid. For the gonad and duct condition of others in this and related lots see Table 3.

The next to be considered in this series of embryos that received 0.25 mg. of

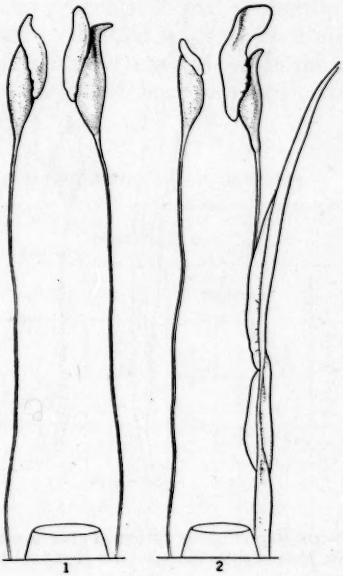

FIG. 1.—Reproductive system of normal 26-day male embryo.

FIG. 2.—Reproductive system of normal 26-day female embryo.

Progynon ranged in age at killing from 18 to 24 days and were injected on the 3d, 4th, 5th, and 8th days. These were from the same lots as the above-described 26-day individuals but were removed at earlier ages. In each instance the minimum interval required for the appearance of cells in synizesis in the cortex had elapsed. Of the twelve cases in this unit, five showed development recognizable as cortex, two of which displayed some cortical cells in synizesis. The best cortex

here was considerably inferior to that described for the 26-day cases.

As to the Müllerian ducts of these twelve cases, only two instances were found in which either duct was entire. The persisting duct was the left, and the Müllerian duct development of these individuals was very much like the normal female. In the others the ducts were either absent, possessed of swollen cystic anterior tips, or had several cysts at intervals, all fluid-filled, as shown in Figure 4, which exhibits the second best-developed case in this group. This case is also interesting in that the thickened epithelium is indicated macroscopically on the surface in both gonads. On the left gonad it is recognizable microscopically as cortex with patches of cells in synizesis; on the right there are thickened areas of epithelium, which are thinner than the cortex on the left gonad. The protocol reveals that this embryo was 22 days old and had been injected on the 4th day.

As regards gonad surface, four other embryos displayed minute ridges over the entire gonad. In one case both gonads were involved; in the others the left gonad only. Microscopic examinaton disclosed that the sexual cords pushed through the forming tunica albuginea, causing the remaining single-celled layer

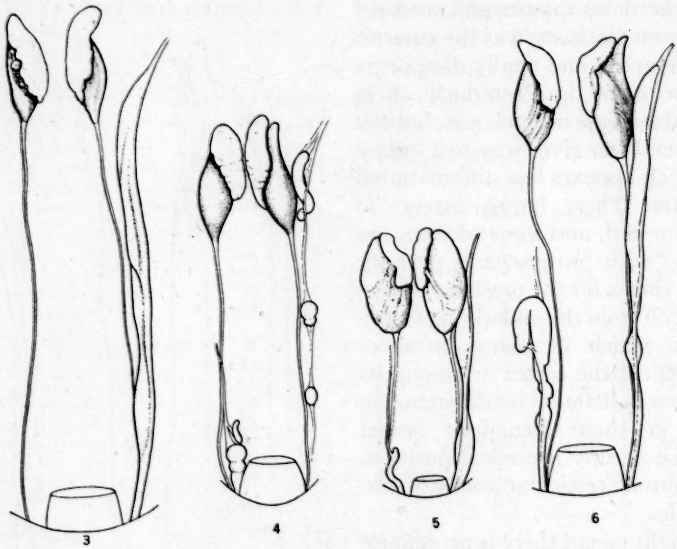

FIG. 3.—Reproductive system of genetic male embryo injected with 0.25 mg. Progynon on 3d day. Age 26 days. Note slightly flattened left gonad (ovotestis), detached lumps of right oviduct, and female-like left oviduct.

FIG. 4.—Reproductive system of genetic male embryo injected with 0.25 mg. Progynon on 4th day. Age 22 days. Note macroscopic evidence of cortical lumps on surface of gonads and the cystic character of the oviducts.

FIG. 5.—Reproductive system of genetic male embryo injected with 0.50 mg. Progynon on 4th day. Age 18 days. Note early modification of gonads and female-like oviducts.

FIG. 6.—Reproductive system of genetic male embryo treated with 0.50 mg. Progynon on 4th day. Age 24 days. Left gonad appears female-like and flattened, right is more rounded. Oviducts are of female type.

to buckle outward. Willier, Gallagher, and Koch (1937) record a somewhat similar phenomenon in the right testis of the chick. They state that the cords not only connect with the free gonad surface but appear to open into the coelom. This ridged condition has been noted in normal duck embryos, but it was not so exaggerated as in the experimental ones.

The youngest killed cases treated with 0.25 mg. of Progynon ranged in age from 10 to 18 days. In an embryo injected on the 5th day, evidence of stimulation of the germinal epithelium was noted as early as the 10th day and was reflected in an increase in the number of germ cells over the normal and in the appearance of incipient cortical cords. Early development of these cords may be traced in embryos 12, 14, 16, and 18 days of age. They may be distributed uniformly or confined to definite spots which are discernible with the unaided eye on the surface of the gonad. Such localized cortical

TABLE 3

SUMMARY OF EFFECTS OF PROGYNON ON GONADS AND MÜLLERIAN DUCTS OF MALES

No. of Embryos	Dosage (Mg.)	Injection Age (Days)	Incubation Age (Days)	Gonads			Müllerian Ducts																	
								Left Inc.†						Left Cystic‡	Right Inc.								Right Cystic	No Ducts
								⅔ §		⅓		½			⅔		⅓		½					
				Differentiated Cortex*	Undifferentiated Cortex	No Definite Cortex	Left Entire	A.‖	P.¶	A.	P.	A.	P.		A.	P.	A.	P.	A.	P.				
5	0.25	3	18–26	1	1	3	1					1	1							1	1	1	2	
6		4	22–26	2	1	3	1							1						2			4	
12		5	10–26	1		11	8**	1				1		1			1		1	2	1	1		
8		8	22–26	2	1	5						3		3					6				2	
2	0.50	3	26	1	1		1	1												2				
32		4	16–25	6	6	20††	14	1		2		4	1	5			2	1	15		5	5	5	
14		5	14–26	6	3	5	4**	1				3		3			3	1		2	3	3		
13		6	14–26	2	1	10††	2			2		5		3			4	2	2		2	1		
18		10	16–8‡‡			18						5		2			1		4		5	7		
21		12	18–2‡‡			21						9		2					5		9	5		
16		14	20–8‡‡			16						4		1					3		1	11		
3		16	22–24			3																3		
11		18	22–27			11						2									1	9		
4	0.75	4, 6	20–22		3	1††	1					2		1		1			1		1			
9	1.00	4, 6	20–26	1	7	1††	2	1		1		2		3				1		4	2			
8	1.50	4, 6, 8	25	3	2	3††	4			1		1		2	1		2	1			4			

*"Differentiated Cortex" refers to cortex with cells in synizesis, and they may be few or many. "Undifferentiated Cortex" indicates cortex lacking cells in synizesis.

†"Inc." = Incomplete.

‡The term "Cystic" in this table refers to fluid-filled enlargements occurring singly or severally, provided they do not occur singly at either anterior or posterior extremity of the Müllerian duct. Cysts found there have been included in the fractional lengths of these respective ends.

§The fractions indicate approximate lengths from either end.

‖"A." = Anterior end.

¶"P." = Posterior end.

**Some too young to show degeneration.

††Not all sectioned.

‡‡Post-hatch age in days.

areas are shown on the gonads in Figure 4. No fully differentiated cortex was observed in any of these ten cases which comprise the youngest category. Study of these earlier stages rules out any possibility of early cortical proliferation and subsequent degeneration prior to hatching.

Müllerian duct development ranged from no unusual development in the 10-day cases, through a regional development, similar to Figure 4, to a complete left duct with posterior vestigial tip on the right in an 18-day embryo. Those male embryos 10, 12, and 14 days old shown in Table 3 with entire left ducts display a duct condition typical of the normal young male embryo before degeneration sets in. The absence of degeneration in the 12- and 14-day-old cases, when regression is normally apparent, may result from individual differences in rate of development or maintenance by the introduced estrogen.

Group II.—Dosage here was 0.50 mg., and the injection age was from 3 to 18 days. In those embryos injected from the 10th through the 18th day this quantity of Progynon had no noticeable effect upon the germinal epithelium; but there was Müllerian duct hypertrophy after 10 days, so these cases will be included only in the discussion relative to Müllerian ducts. This leaves forty-eight male embryos injected between the 3d and 10th days for consideration, with an age range of from 18 to 26 days. Two embryos injected on the 3d day and killed on the 26th day showed sexual cords protruding individually and collectively from the surface of the left gonad. They may project alone or anastomose, forming a cap readily distinguished from the rest of the gonad. In this cap in one of the embryos, a layer of cells in synizesis increased to a depth of two cells in places. The other did not show this differentiated layer but the cap of cords was definite. The condition really suggests the proliferation of a lump of cords on the surface of the gonad from underlying testis cords, which lump is endowed with the ability to form cortex. The surface of the left gonad in treated individuals frequently exhibits a pronounced corrugated appearance, which in some instances may be indicative of this condition.

In many cases observed in this investigation the character of the thickened outer layer on the left testis in embryos of from 20 to 26 days' incubation has the aspect of having been formed by outgrowing testicular cords rather than by cords formed in the epithelium. Figure 32 shows a cap of cortex with connecting testicular cords. When this cap is traced anteriorly, it covers a larger area of the gonad and shows cells in synizesis. Figure 31 illustrates an early stage in the outgrowth of testicular cords which form surface lumps.

Thirty male embryos were examined in the 4-day-old injection group, eighteen of them microscopically. These eighteen were sectioned because gross examination revealed more variation from the normal in gonad and Müllerian duct formation than in the others.

The effect of Progynon was strongly registered upon the gonads of this group in macroscopic appearance. The lateral margins of both right and left gonads were decidedly irregular in outline, showing flattened, finger-like projections, as described above, extending over the Wolffian body, which in some instances terminated in detached lumps; or there might be detached lumps along the lateral border occurring with greater frequency toward the posterior end (see Fig. 3). These projections or detached

lumps, when sectioned, proved to be testicular in character. The right gonad usually appeared broader from the ventral view and did not show a marked tendency to become rounded, testis-like, as observed by Willier *et al.* (1937) and others, in the chick. The left was always longer, as Figures 5 and 6 reveal; yet occasionally it was smaller in diameter. This disparity in length was approximately as much as one-third in some cases.

Only two of the eighteen embryos sectioned showed a marked differentiation of the cortex, well delimited from the underlying cords, which were testicular in nature. These were killed on the 25th day. The cortical tissue did not cover the entire ventral surface of the gonad in either case but was applied caplike, with both medial and lateral margins uncovered. It extended almost the length of the gonad also, but anterior and posterior tips were bare. In one case the maximum thickness approximated closely the normal of comparable age but was decidedly lacking in the extent of this thickness. Many of the cells in these two cases were in synizesis.

The other sixteen individuals displayed all the intermediate stages, from those just described to no noticeable stimulation. Some of these showed a thin layer, sometimes one cell in thickness, of cells in synizesis in places, while others showed a rather thick layer, not well marked off from the underlying tissue, which had the appearance of cortical tissue in the presynaptic stage. The ages at which this group was killed ranged from 18 to 25 days (see Table 3 for complete record). One case killed on the 18th day showed an early stage in cortex formation from the germinal epithelium (Fig. 23). The gross picture of the genito-urinary system of this individual is shown in Figure 5.

Müllerian duct development was good in this lot. Figures 5 and 6 show hypertrophy throughout in the left duct, with a variable degree of hypertrophy on the right. Fourteen of the thirty embryos showed development of the left duct in its entirety, with the right persisting from more than half its length to not at all; five showed no Müllerian ducts. In the other cases the persisting parts were cystlike, which occurred singly or several to a duct in tandem fashion at any level. The higher dosage of hormone administered in Group II evidently accounts for the superior duct development over Group I.

The next lot under Group II was injected on the 5th and 6th day. Seventeen embryos comprise this group, and sixteen were sectioned for microscopic study. On the whole, cortex development was not so good here as in the embryos injected on the 4th day. Eight showed a fair degree of cortical differentiation, but all were inferior to the 4-day injected embryos in this regard. In four others, cortical differentiation had proceeded only far enough to be recognizable as such. The gross contour of the gonads did not appear so greatly altered either, as Figures 7 and 8 reveal. There is some irregularity along the margins, accompanied by detached lumps and a slight disparity in size but not so exaggerated as in the previous lot. Figure 8 shows a flattening of both gonads, giving an ovary-like appearance.

In five embryos of this category with the best-developed cortex, this tissue extended over most of the gonad surface, being thicker in all instances toward the caudal end. In places, especially craniad, it was not well delimited from the underlying tubules. All these embryos were 26 days old when killed, except one 24-day embryo, which had as thick a cortex but

not so many cells in synizesis. Three of these birds were injected on the 5th day and two on the 6th day. The 6-day cases were about as good as the 5-day. The other three embryos with differentiated cortex not so well developed had been injected on the 5th day and were 22 days old. The gonads represented in Figure 7 are from one of these 22-day-old embryos whose cortex was too poorly developed to be ranked among the first five. It consisted of a very thin layer, with a few differentiated cells here and there. Killing age might have been a responsible factor. On the whole, development of cortex did not appear to measure up to the standard of the embryos treated on the 4th day. However, this is not absolutely conclusive evidence, although it suggests that Progynon may not be so effective when injected on the 5th or 6th days as when injected on the 4th, because there were many more cases to judge from in the 4-day injected category.

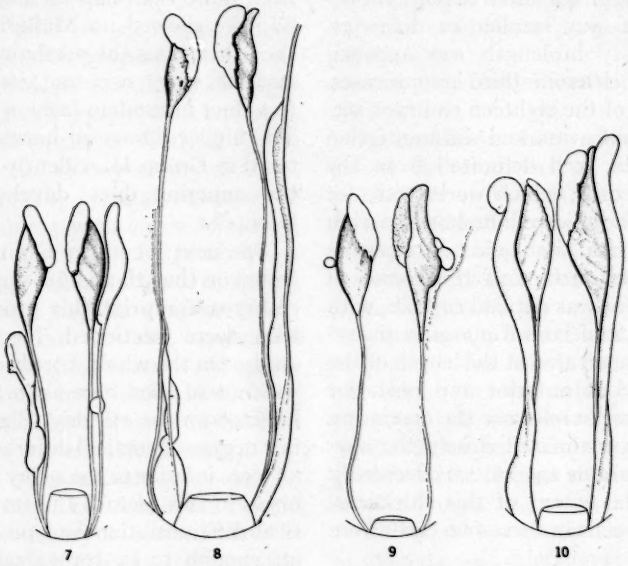

FIG. 7.—Reproductive system of genetic male embryo treated with 0.50 mg. Progynon on 5th day. Age 22 days. Note greater length of left gonad and character of oviducts.

FIG. 8.—Reproductive system of genetic male embryo treated with 0.50 mg. Progynon on 5th day. Age 26 days. This shows a good intersexual condition.

FIG. 9.—Reproductive system of genetic male embryo injected with 0.50 mg. Progynon on 12th day. Age 22 days. Note corrugated condition of left gonad, also persisting anterior ends of oviducts.

FIG. 10.—Reproductive system of genetic female embryo of same age and treatment as shown in Fig. 9. Contrast the well-developed right oviduct with those of the male.

Müllerian duct development, on the whole, was good in the above lot. The left duct was entire and female-like; practically entire, as illustrated in Figure 8; or entire with enlargements connected by slender portions, as shown in Figure 7. There were seven of these. The size in the others ranged from these conditions to merely a persisting anterior tip. The best right Müllerian duct development is

also shown in Figures 7 and 8. In four specimens it was absent.

Ten other embryos injected on the 5th and 6th days, whose ages at killing were between 14 and 18 days, showed varying degrees of cortical differentiation and duct development. They are not included in the discussion because the time elapsed at killing was under the minimum at which fully differentiated cortex had been observed, and, with but two exceptions noted below, none revealed development of cortex. One of these individuals injected on the 6th day, and 18 days old when killed, had a thickened epithelium with a suggestion of cortical cords in places. Another 16-day embryo, injected on the 4th day, appeared more like normal untreated embryos with a lumpy condition of the germinal epithelium.

In an attempt to determine just how late the germinal epithelium is modifiable by Progynon, embryos were injected as late as the 18th day of incubation. Embryos in this division of Group II, receiving 0.50 mg. Progynon, ranged in age from 10 to 18 days. Because of the late age at which the hormone was administered, a 14-day interval between treatment and killing did not elapse in all cases. A total of sixty-nine male embryos in this category was examined macroscopically, thirty-nine of which were sectioned and studied.

The gross structure of the gonads of these individuals was not greatly altered. In some instances there was a suggestion of flattening, which was not particularly striking. The margins varied from a smooth through a serrated condition to the previously noted small, finger-like projections. The right gonad was usually shorter, but not much so; and it was sometimes broader, as Figure 9 portrays. This same figure shows the rough corrugated surface on the left gonad alluded to before. This was observed in fourteen of the sixty-nine embryos injected from the 10th to the 18th day and killed from the 18th to the 28th day. It appears to result in large measure from primary sexual cords pressing against the surface and small lumps of persisting germinal epithelium.

There were numerous instances in which either a rather thick, almost continuous, outer layer or lumps more widely separated invested the sexual cords; but these structures were more similar in nature to the thickened outer layer described as persisting on the normal left testis than to differentiated cortical cords. One case was injected on the 10th day, and 10 days later, when 20 days old, it possessed a layer on the left gonad that was not unlike that found on the left gonad of a 10-day old untreated normal male embryo. One, injected on the 12th day and killed when 24 days old, showed a thickened layer that had the appearance of incipient cortical cords; but this was too doubtful to be recorded as certain. In a few cases the persisting patches of cells consisted principally of germ cells in groups of one to four or five. In eight cases all traces of the epithelium had been lost. This entire lot, injected from the 10th to the 18th day, can be said, therefore, to show no positive response, as far as cortical development is concerned.

With the Müllerian ducts a slightly different story is told. Stimulation here is not great but definite and in keeping with what might be expected from normal development, that is, persistence is greater at the anterior ends, where the final stage of degeneration of the Müllerian ducts in the male occurs. Among the sixty-nine cases in this lot, thirty-two showed remnants of ducts. In no speci-

men was the duct entire but, in the great majority of cases, consisted only of the anterior tips, as shown in Figure 9 (for summary, see Table 3). These results indicate that the ability to respond to hormone treatment is retained longer by the ducts than by the germinal epithelium covering the testes. Oviduct response was more pronounced in the female than in the male, as Figures 9 and 10 disclose. The two embryos illustrated were from the same lot of eggs, are the same age, and had the same treatment.

Group III.—These embryos received 0.75 mg. of Progynon. This was administered by injecting 0.25 mg. on the 4th day and 0.50 mg. on the 6th. The mortality rate was high, and from the thirty-two eggs originally injected, only four males and five females survived at the end of the 20th, 22d, and 26th days. No males were living on the 26th day, and the protocol states that, of the eight eggs removed at this time, three modified males and two females were dead and three females survived.

The two males killed on the 20th day revealed flattened gonads with irregular margins, and the left gonad of one showed protuberances on the surface. The two killed on the 22d day had rather slender, flattened gonads.

Gonads from three of the four males were studied histologically. The increased dosage exercised surprisingly little effect, for, at these ages, none showed a fully differentiated cortex. In all three cases young cortical cords were definitely in process of formation but had not attained the degree of development commensurate with the age and dose administered, since some of the cases described reveal better differentiation at 22 days with only 0.25 mg. Progynon injected on the 4th day. The difficulty here may rest with the small number of cases; yet the fourth unsectioned case did not appear sufficiently modified to warrant sectioning.

The increase in dosage was readily apparent in the Müllerian ducts. One of the four males comprising this group had a left duct of normal length, greatly distended at the anterior end and hypertrophied at the posterior end. The right duct was large at its cranial end, as was the left, but it originated slightly anterior to the caudal end of the Wolffian body and extended to the cloaca. None of the other three had a complete duct on either side (see Table 3 for summary).

Group IV.—Dosage in this group of embryos was 1.00 mg. of Progynon administered on the 4th and 6th days in 0.5-mg. quantities. Nine individuals were studied macroscopically, and eight of these were sectioned. Age at killing ranged from 20 to 26 days. Grossly considered, the left gonad showed considerable evidence of posterior flattening in all cases; the flattening extended more anteriorly in five cases. In contour the margins of both gonads were irregular, flanked by detached lumps in some cases, and the surface of the left usually exhibited a lumpy condition. There was a disparity in size similar to the normal female in three instances, and in others one or both gonads were slender and elongated or not greatly altered.

The best cortical differentiation did not equal that of some embryos that received only 0.50 mg. Every individual exhibited a layer of variable thickness superimposed upon the testis cords, but its manner of origin appeared peculiar in some instances. Indications point to an outpushing of primary testis cords to the surface, followed by a multiplication of these on the surface (Fig. 33, Case 16P4622b, and Fig. 28, Case 16P4626c) which later become cut off to form a sec-

ond layer (Fig. 29). There are sections which show a single cord projecting alone and others that show this layer connected by tubules to those in the inner portion (Fig. 33). Perhaps such a method of formation requires a longer period and therefore accounts for the lack of fully differentiated cortex in this outer layer which was found in some treated embryos of a comparable age.

Only one case showed thickening that displayed differentiated cells, and here differentiation was incipient. Its cortical layer extended practically over the entire surface of the gonad. A transverse section showed the layer to be fairly thick on the medial surface but extremely thick at the margins of the hilus. There was a pronounced line of demarcation between the two layers, which became accentuated posteriorly, resulting in two layers of equal size, composed of cords of very different appearance (Fig. 29, Case 16P4626c). Although the outer layer was well developed, there was an absence of cells in synizesis at this level. Farther anteriorly, however, it showed a patch of fully differentiated cortex (Fig. 30). Figures 28, 29, and 30 are from the same embryo at different levels and apparently show testicular cords growing out, becoming cut off, and differentiated into cortex. As a rule, complete differentiation of the cortex begins anteriorly and proceeds posteriorly where it is thickest.

This group of embryos appears to furnish further proof that cortex may develop from young testicular tubules, as referred to earlier. A detailed study of all cases in our investigation seems to disclose two methods of cortical formation, namely, one from cortical cords formed from the germinal epithelium and the other from testicular cords, which grow out and become detached from the underlying tubules. In the latter method a longer period is required for cells to appear in synizesis.

Müllerian duct development showed an improvement over Group III. Two of the nine in the lot showed development of the left duct throughout its length, and both of these displayed fluid-filled cystic enlargements at the posterior end of the right instead of the anterior. This latter condition is in keeping with normal development in the female (details of others are in Table 3).

Group V.—In this group the dosage was stepped up to 1.50 mg. This was given on the 4th, 6th, and 8th days in 0.50-mg. portions. All the embryos in this lot were killed on the 25th day, as experience had shown mortality on the 26th day, just prior to hatching, to be very high. Eight males survived for study, five of which were sectioned for histological details. Four of the eight showed a decided disparity in gonad size in favor of the left. In the other four, the left gonad was larger, too, but not so markedly. Considerable flattening was evident in the left gonad, as well as a lumpy surface, in all eight embryos of this group. The right was a bit more rounded in contour, but the margins were more irregular.

The microscope disclosed that the left gonads of three of the five embryos sectioned had pronounced cortical development. Among these, one had a very thick cortex, but most of its cells were in presynaptic stages, and there appeared to be a number of connecting cords between it and the primary sex cords. The other two cases showed cells in synizesis, hence were further along in differentiation. The remaining two of the five cases had a very thick layer of cells, containing some primordial germ cells, which was confined chiefly to the posterior half of the gonad

and did not extend across the breadth even here.

On the whole, Müllerian duct development was pronounced in this group. The left duct was either entire and quite large (four cases), large at the anterior end with intermittent cysts in the posterior half (two cases), or huge anteriorly with little or no hypertrophy at the posterior end (two cases) (see Table 3). The ducts of one of the latter were so large that they obscured the gonads and Wolffian bodies. All individuals of this group showed decided Müllerian duct enlargement. This, coupled with the fact that the best cortical development among the Progynon-treated birds was found in Group V, indicates that it is possible by increase in dosage to augment cortical differentiation. However, the augmentation with a dosage of 1.50 mg. over that following a dosage of 0.25 mg. did not appear to be commensurate with the increase in quantity. The Müllerian ducts were much more responsive to this stepped-up dosage.

2. *Females: Group VI.*—This group is comprised of the one hundred and seventy-five females treated with Progynon (0.25–1.50 mg.). While all these were examined macroscopically, only selected cases were studied microscopically. The principal effect of the hormone was registered upon the oviducts in the female, and a total of fifteen was selected for sectioning because they had either a large right rudiment, a very small right rudiment, or unusual Müllerian duct formations. These cases varied as to time of injection, age when killed, and dosage and are believed to give a good indication of the general effect of this hormone in the female.

The vast majority of the embryos reflected little or no change in ovarian structure as a consequence of Progynon treatment. However, the most pronounced cortical development on the right ovary was observed in two individuals that received 0.50 mg. of Progynon on the 4th day and were killed on the 18th day. No female among the eleven normals studied histologically showed any cortical growth on the right gonad comparable to the condition found in these two cases. Indeed, one of these individuals showed development of cortex superior to that on the left ovary at the same level. It was a continuous layer plainly demarked from the underlying medulla and was as thick as this medulla. Macroscopically, the individual under consideration had shown a short, plump right gonad near the anterior end of the left, which ended abruptly, posteriorly. The other case was as thick as this in places but was not so fully differentiated, and the cortex occurred more in lumps than continuously.

Another female, which received 0.50 mg. of Progynon on the 4th day and was killed on the 25th, revealed the best development of cortex on the left found in all the embryos studied in this investigation. At about the mid-point in cross-section, the cortex of the left gonad at the lateral border of the hilus was enormous and appeared augmented by an extra lobe wedged in ventrolaterally. The ratio of the area of medulla to cortex in this section was approximately 1:5. The right rudiment in this embryo was unusually large and in cross-section displayed no well-defined cortex but a mass of lumps that appeared more like cortex than like medulla. Some of these lumps at various depths showed cells in synizesis. One other female, which had received 0.25 mg. on the 4th day and had been killed on the 26th, showed cortical development similar to this case, but it was not quite so pronounced. The right

gonad here was unusually large also and displayed a better-defined cortical area than did the preceding individual.

These four cases suggest some cortical stimulation in females as a consequence of estrogen treatment but by no means prove it. We realize that they may simply be individual variations. In one paper, Dantchakoff (1935*b*) postulates that Progynon in females acts as a whiplash to speed up histogenetic differentiation with more vigor and rapidity. This may apply to some extent in the duck.

The Müllerian ducts of females appear to respond to Progynon in direct proportion to the dose and in inverse proportion to the age at the time of injection. Of twenty-seven cases aged 16 days and above, receiving 0.25 mg. at ages of 3–8 days, only six showed slight hypertrophy of the left duct and persistence of the right from the cloaca to the posterior level of the Wolffian body. Four others showed a posterior enlargenent of the right duct at the cloaca. The general rule for the persistence of the right duct, as might be expected, is that hypertrophy occurs most often in the cloacal region and extends to higher levels in a decreasing number of instances.

As dosage is stepped up, there is an augmentation in diameter of the left duct and of length and diameter in the right with the younger injection ages (2–8 days). The highest dosage (1.50 mg.) produced the largest ducts, and both were entire in some females (three out of five), or both were much enlarged, with the right extending only about halfway (two out of five) (see Table 4).

Just how late the highest dosage of Progynon is capable of causing tumescence of the oviducts in females was not ascertained beyond question; but 0.50 mg. can cause a pronounced enlargement practically throughout the length of the right and left ducts as late as the 12th day (Fig. 10). This amount was injected as late as the 18th day; and, of eight females, one showed slight hypertrophy of the left, and at the cloaca the right was somewhat enlarged; one showed slight enlargement in the left only; three revealed enlargement of the right posterior tip only; and three exhibited no hypertrophy of either. It may be noted in passing that, since it is about the 18th day that normal oviduct differentiation is complete, it is doubtful whether hormone administration following the 18th day would produce any noteworthy effect other than hypertrophy in the Müllerian ducts. A summary of the effects of Progynon upon the Mülleran ducts in females is given in Table 4.

B. RESULTS OBTAINED WITH STILBESTROL

Since Progynon failed to bring about complete sex reversal, it was decided to try other estrogens. Those utilized were diethylstilbestrol and hexestrol. These hormones are much more soluble in sesame oil, hence higher concentrations in the same quantity of fluid were possible. In the case of Progynon, $\frac{1}{20}$ cc. of solution contained only 0.25 mg. of hormone, whereas the same amount of sesame-oil solvent might contain 1.25 mg. or more of stilbestrol and hexestrol. Stilbestrol in the concentration used proved very toxic; hence only one lot of eggs was treated with this hormone. Table 5 summarizes some of the data obtained on the effects of stilbestrol.

1. *Males.*—Forty-five eggs were injected with 1.25 mg. of stilbestrol on the 4th day of incubation. The plan was to kill these embryos on the 26th day; but candling from time to time revealed so many dead embryos that on the 22d day, when only four remained alive, it was decided to kill these for fear that they

TABLE 4

EFFECTS OF PROGYNON ON MÜLLERIAN DUCTS OF FEMALES

No. of Embryos	Dosage (Mg.)	Injection Age (Days)	Incubation Age (Days)	Left Duct								Right Duct							
				Entire		⅓*		⅓		⅓		Entire		⅓		⅓		⅓	
				Hyp.†	Nor.‡	A.§	P.‖	A.	P.	A.	P.	Hyp.	Nor.	A.	P.	A.	P.	A.	P.
5	0.25	3	26	.....	5	...	...	...	...	...	...	....	....	...	...	...	...	...	5
10	.....	4	22–26	.....	10	...	...	...	...	...	...	....	....	...	...	...	...	...	10
17	.....	5	10–26	2	15	...	...	...	...	...	...	....	4	...	4	...	4	...	5
8	.....	8	22–26	4	4	...	...	...	...	...	...	....	....	...	2	...	1	...	5
30¶	0.50	4	16–26	11	12	2	2	1	...	...	1	....	1	...	2	...	2	1	15
7	.....	5	14–26	3	4	...	...	...	...	...	...	....	3	...	1	...	2	...	1
17	.....	6	14–26	13	4	...	...	...	...	...	...	4	3	...	5	...	3	...	2
12	.....	10	18–8**	12	.....	...	...	...	...	...	...	2	.....	...	8	...	2	...	...
22	.....	12	18–2**	21	1	...	...	...	...	...	...	1	1	...	4	...	6	...	10
17	.....	14	20–8**	6	11	...	...	...	...	...	...	....	....	...	....	...	....	...	17
11	.....	16	22–28	2	9	...	...	...	...	...	...	....	....	...	....	...	....	...	11
8	.....	18	22–27	2	6	...	...	...	...	...	...	....	....	...	....	...	....	...	8
5	0.75	4 and 6	20–26	5	.....	...	...	...	...	...	...	....	....	...	2	...	...	...	3
2	1.00	4 and 6	20–26	1	.....	...	...	...	...	1	...	....	....	...	1	...	...	...	1
5	1 50	4, 6, and 8	25	5	.....	...	...	...	...	...	...	3	....	...	....	...	2	...	...

* The fractions indicate approximate lengths from either end.

† "Hyp." = Hypertrophied.

‡ "Nor." = Normal.

§ "A." = Anterior end.

‖ "P." = Posterior end.

¶ One has no left Müllerian duct.

** Post-hatching age in days.

TABLE 5

DATA ON EFFECTS OF STILBESTROL

Injection Age*	Quantity Injected (Mg.)	No. of Eggs Injected	Embryos Killed				Survival (Percentage)
			Age*	No.	Male	Female	
4	1.25	45†	22	4	2	2	8

* Incubation days.

† The discrepancy between number of eggs injected and embryos killed is due to the death of the embryos in the interim.

would not survive until the 26th day. Examination revealed two males and two females.

One of the males displayed gonads with finger-like processes extending from the lateral margins, a condition similar to that observed when Progynon was administered. The left gonad was somewhat flattened and more ovary-like, while the right was more rounded. In the other male the gonads were very slender and elongate. The former male was chosen for sectioning because gonads similar to those of the latter, treated with Progynon, had been sectioned and showed scant cortical development.

A microscopic study of the gonads of the male disclosed a cortex on the left somewhat thinner than in normal females, with a few patches of cells in synizesis and flattened lumps of thickened epithelium at intervals on the right. The cortex on the left gonad covered most of its surface but, as usual, was thicker posteriorly and thinned out toward the anterior end.

Both males had huge ducts also; however, the enlargement did not extend all the way to the cloaca—only about two-thirds of the distance in the longer. Figure 12 shows the shorter of the two.

2. *Females.*—In the females, one right rudiment was large and one was small. The left ovaries showed nothing unusual in appearance. Figure 11 shows the female with the large right rudiment.

The ovary of the female with the larger rudiment, when sectioned, exhibited an unusually thick cortex, especially on the lateral border as seen in cross-section, which was reminiscent of the condition of the two Progynon-treated females discussed in Group VI. Most of the cells were in synaptic stages, except those on the median and the lateral borders. The right rudimentary gonad, although larger than usual, did not possess a well-defined cortex but contained cortical and medullary cords not clearly demarked. Gonads of the other female were not sectioned.

The illustrations show that the stilbestrol-treated embryos noticeably surpassed the Progynon-treated ones in Müllerian duct development. Comparison of Figure 10 (best-developed Progynon case) with Figure 11 gives an idea of the difference. Both of these were drawn to the same scale and were 22 days old. The tumescence is more exaggerated in Figure 11. The second female showed ducts just about as large as this one.

C. RESULTS OBTAINED WITH HEXESTROL

Since hexestrol proved less toxic than stilbestrol, as indicated by a lower mortality, further observations were continued on the effects of hexestrol administration.

1. *Males Group I.*—For these embryos an attempt was made to put 25 mg. per cubic centimeter of hexestrol into solution in sesame oil. When this failed, the concentration was reduced to 12.5 mg. per cubic centimeter; but even with this dilution all the solute was not completely dissolved, and a fine suspension still pervaded the solution. This was used but was thoroughly agitated just prior to each injection. The quantity given was $\frac{1}{10}$ cc., which should have been equivalent to 1.25 mg., however, since the hormone was not completely in solution, there existed the possibility of variability of dosage. The number of surviving male embryos was five, two of which were killed on the 24th day and three on the 26th day.

The gonads of these five male embryos bore evidence of strong hormonal influence. In all cases there was considerable difference in size in favor of the left. Both

gonads had very irregular lateral margins and were somewhat flattened, especially the left, which also was broader in most of the cases. The urinogenital system of one of these interesting cases is shown in Figure 13. This illustrates about as great a disparity in size of gonads as was obtained.

The most complete cases of reversal in this study, as shown histologically, were found in two individuals of this group. One, killed on the 26th day (illustrated in Fig. 13), possessed a cortex on the left testis as fully differentiated and as thick as the untreated female (Fig. 25). This differentiated cortex extended from about the middle of the gonad to the posterior end. The underlying tissue was definitely testicular in character, as the illustration shows. Figure 26 shows an enlarged section of this cortex with cells in synizesis similar to those of the normal female, shown magnified more highly in Figure 17. From the mid-point anteriorly, the cortex became thinner, terminating in a scant layer at the anterior end of the gonad. The cortex on the caudal half was set off from the under-

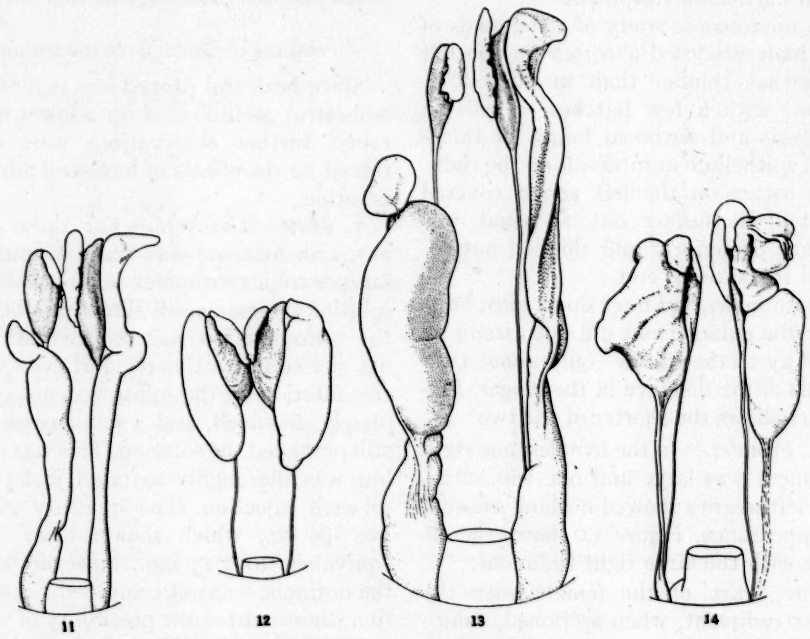

FIG. 11.—Reproductive system of genetic female embryo treated with 1.25 mg. stilbestrol on 4th day. Age 22 days. Note large right rudiment and compare duct development with Fig. 10.

FIG. 12.—Reproductive system of genetic male embryo of same age and treatment as Fig. 11. Compare duct development with Fig. 9.

FIG. 13.—Reproductive system of genetic male embryo injected with 1.25 mg. hexestrol on 4th day. Age 26 days. Note disparity in gonad size and enormous oviduct development. This represents the most highly modified condition obtained in males.

FIG. 14.—Reproductive system of genetic female embryo of same age and treatment as Fig. 13. Observe the enormous development of the anterior ends of the oviducts to the exclusion of the posterior.

lying tubules by connective tissue. There was no evidence of medullary cords of the female type, those underneath the cortical layer being clearly recognizable as testicular in nature. The entire urinogenital system of this individual is shown in Figure 13.

The surface of the right gonad showed fairly thick lumps of epithelium in places. Very remarkable, however, was the appearance of a layer of cells, some approaching synizesis, on the median dorsal surface of the right Wolffian body, some distance posterior to the terminal portion of the right gonad. It was definitely cortical in appearance.

The other male embryo which displayed a high degree of reversal belonged to the same lot as the one just described, had the same dosage, and was killed at the same age. Macroscopically, the right and left gonads also showed about as great a disparity in size. Although the cortex on the left gonad was practically as well developed, the outstanding difference was the relationship existing between the cortex and the rest of the gonad. The former gave the appearance of something superficially imposed or tacked onto the medulla, with considerable connective tissue between (see Fig. 25). This relationship did become somewhat more intimate toward the cranial end of the gonad, whereas, in the latter individual, the cortex appeared to have the same relationship to the underlying portion as the normal female cortex has to the underlying medulla (Fig. 27). This underlying area, however, was not typically female. Two types of tissue were distinguished, namely, connective tissue composed of elongated, flattened cells, and testicular tubules.

The three remaining male embryos in this group showed lesser grades of gonad transformation. The 26-day male, which had unusually long, thin, slender gonads, with the left much longer than the right, exhibited only a thin layer of cortex on the left gonad in cross-section. The layer was a bit thicker at the caudal end but not unusually so. The other two were killed on the 24th day. One displayed good cortical development, but it was not on a par with the best. The second 24-day embryo possessed cortex of only medium thickness on the left gonad, with fairly numerous groups of cells in synizesis.

Müllerian duct hypertrophy was so enormous that it approached the bizarre (Fig. 13). The ducts were huge in all cases and were either entire on both sides (three cases), entire on the left and almost so on the right (one case), or ballooned only at the cranial end on both sides (one case). In one of these birds the ducts obscured the gonads entirely, making dissection of them without injury extremely difficult. Duct development following hexestrol administration surpassed by far even that produced by stilbestrol (see Table 7).

Group II.—This group will be considered in two lots—those injected on the 4th, 6th, or 8th day to determine to what extent the germinal epithelium may be modified by heavy dosage of hexestrol and those injected at from 9 to 18 days to ascertain the period at which induction of cortex is no longer possible. The dosage for this group was 2.50 mg.

The first lot consisted of twenty-seven male embryos, which were killed from the 19th through the 26th day. It was originally intended that they should be taken on the 26th day, but the high rate of mortality made it necessary to change to the 25th day or earlier. Table 6 shows the survival percentage to be exceedingly low.

Gross study showed that all ten of the

male embryos injected on the 4th day possessed gonads dissimilar in size, a condition which was obvious at a glance. It was the left gonad that was larger, and very decidedly so in four cases. Often the gonads were so crowded because of tumescence of Müllerian ducts that the shape was not readily apparent; but, in one end of the left gonad to the other, and one showed cells in synizesis at the posterior tip, which has been noted only in a high-grade intersexual type. Two had cortex equivalent to the poorest developed in normal females of the same age, and these gonads might readily have been taken for ovaries, had it not been

TABLE 6

DATA ON EFFECTS OF HEXESTROL

Injection Age*	Quantity Injected (Mg.)	No. of Eggs Injected	Embryos Killed				Survival (Percentage)
			Age*	No.	Male	Female	
4	1.25	45	24–26	21	5	4	20
4	2.50	145	25	33	6	2	11
6		45	19–25	27	9	5	31
8		30	19–25	17	8	6	46
9		25	23–27	12	8	3	44
10		29	25–28	17	4	1	17
12		53	22–2†	35	12	13	36
14		62‡	25–4†	44	11	7	29
16		30	28–2†	8	5	3	26
18		16	27–4†	15	2	2	25
4 and 6	3.75	60					(All dead by 10th day)
Totals of sexes					52	37	
Sex ratio (per cent)					58	42	

* Incubation days.

† Post-hatching.

‡ The discrepancy between number of eggs injected and embryos killed in all cases in the table is due to to the death of embryos in the interim.

general, the left was flatter, and the right, which had a regular lateral margin, was only slightly more rounded.

The gonads of nine of the ten male embryos were sectioned and studied further for histological changes. In five embryos the left gonads showed transformation of the same general quality and were decidedly of a high-grade intersexual type but somewhat inferior to the two described in Group I, with regard to amount of cortex. In all five cases there was good development of cortex from for the unmistakable male character of the underlying tubules. The right testis in none of these individuals developed a definite cortical layer.

The other four cases showed varying lesser degrees of cortical thickness on the left gonad, but all exhibited at least a few fully differentiated cells. Reference to Table 7 will disclose that the dosage employed was instrumental in producing cortex that showed cells in synizesis in every treated male in Group I and those injected on the 4th day in Group II. De-

spite this fully differentiated cortex on the left testis in all cases, there was no transformation of testicular cords into medullary cords—hence no complete inversion. No definite cortical layer was found on the right testis.

The other individuals comprising lot No. 1 of Group II were those injected on the 6th day (nine cases) or the 8th day (eight cases). Two of the males treated on the 6th day showed fairly thick, fully differentiated cortex only slightly inferior to those injected on the 4th day, and five other males displayed undifferentiated cortex on the left gonad. Among those treated on the 8th day, fully differentiated cortex on the left gonad, not quite so good as that shown by those injected on the 6th day, was found in two individuals and undifferentiated cortex in four (see Table 7 for ages, etc.).

Hypertrophy of Müllerian ducts in this lot was exceedingly great also. Six of the ten injected on the 4th day had enormous, full-length oviducts on both sides (see Table 7 for greater details). These ducts were so large that they often covered the gonads entirely and, coupled with the viscera, sometimes pressed them into abnormal shapes.

TABLE 7

SUMMARY OF THE EFFECTS OF HEXESTROL ON GONADS AND MÜLLERIAN DUCTS OF MALES

No. of Embryos	Dosage (Mg.)	Injection Age (Days)	Incubation Age (Days)	Gonads			Müllerian Ducts														
								Left Inc.†							Right Inc.						
								‡‡		½		¼			¾		½		¼		
				Differentiated Cortex*	Undifferentiated Cortex	No Definite Cortex	Left Entire	A.§	P.‖	A.	P.	A.	P.	Right Entire	A.	P.	A.	P.	A.	P.	No Ducts
5....	1.25	4	24–26	5	.....	.....	4	1	...	...	...	...	...	4	1	...	...	...	...	...	...
10....	2.50	4	25–26	10	.....	.....	7	1	...	2	...	...	...	6	...	1	3	...	...	...	...
9....	.....	6	19–25	2	5	2¶	2	4	...	3	...	...	...	...	5	...	3	1	...	...	...
8....	.....	8	19–25	2	4	2	...	1	...	2	...	5	...	...	...	...	1	...	7	...	...
8....	.....	9	23–27	2	1	5	...	1	...	3	...	4	...	...	...	...	2	...	6	...	...
4....	.....	10	25–27	.....	1	3	...	1	...	1	...	2	...	...	1	...	1	...	...	...	...
12....	.....	12	22–2**	.....	.....	12¶	...	...	...	4	...	8	...	...	1	...	3	...	8	...	...
11....	.....	14	25–4**	.....	.....	11¶	...	...	...	...	...	6	...	...	...	...	...	...	6	...	5
5....	.....	16	28–2**	.....	.....	5¶	...	...	...	...	...	2	...	...	...	...	...	...	2	...	3
2....	.....	18	27–4**	.....	.....	2¶	...	...	...	...	...	1	...	...	...	...	...	...	1	...	1

*"Differentiated Cortex" refers to cortex with cells in synizesis and they may be few or many. "Undifferentiated Cortex" indicates cortex lacking cells in synizesis.

†"Inc." = Incomplete.

‡The fractions indicate approximate lengths from either end.

§"P." = Posterior end.

‖"A." = Anterior end.

¶ Not all sectioned.

** Days post-hatching.

Müllerian duct development in embryos injected on the 6th or 8th day was not quite so good as that of those injected on the 4th day, as comparison in Table 7 will show.

The second lot in this group received a dosage of 2.50 mg. of hexestrol either on the 9th, 10th, 12th, 14th, 16th, or 18th day. A total of forty-two males was studied (see Table 7 for distribution and age when killed).

Macroscopically, the testes did not

show any striking deviation from the normal. There were three cases among the 9-day injected embryos in which the left testis appeared a bit larger than the right, more so than in untreated birds, where there is usually a slight disparity in size in favor of the left. Also, one 10-day treated and one 14-day treated embryo exhibited this abnormal size difference. The surface of the gonads varied from a more or less smooth surface through fine projections to coarser lumps, which never attained the broad proportions characterizing good cortical development. There was slight flattening of the left in a few instances, but for the most part it was typically rounded.

Of this lot, thirty-four were sectioned for evidence of cortical differentiation on testes. Three of the embryos injected on the 9th day and killed on the 24th or 27th day showed cortex recognizable as such on the left testis but not especially well developed. Two of these showed some cells in synizesis. It was in these that the left gonad showed up definitely larger than the right and that the lumps on the surface were largest. Those that possessed the finer elevations showed primary sex cords that grew out to the surface in places and sometimes protruded into the coelom. These protrusions appear to account for the finer surface elevations.

Only one of the four embryos injected on the 10th day displayed what appeared to be cortex on the left testis. Its left gonad before sectioning was characterized by the larger-sized lumps. This cortical development was poor in contrast to that of embryos injected with a similar concentration at an earlier age, 4–8 days, with an equal time interval elapsing between injection and killing.

Since Mülleran duct degeneration is not complete before the 18th day, it was decided to extend the injections to that day in order to determine how late they could be modified by hormone administration, as well as to determine when induction of cortex stops. After the 10th day it appears that cortex is no longer developed from the germinal epithelium. This conclusion is based on the results of the study of thirty embryos injected from the 12th to the 18th day of incubation (see Table 7). Some of these embryos, however, showed an outgrowth of testicular tubules in the left gonad to the surface, where they became cut off to form lumps resembling cortex. Figure 34 illustrates one of the best-developed cases and is taken from a 28-day embryo injected on the 14th day. Whether such lumps would have differentiated into cortex if allowed to continue to develop was not determined. It appears significant, however, that these tubules should develop most frequently and to a greater degree on the left testis and should appear only occasionally on the right, where, when found, they are poorly developed.

It appears that, as Müllerian duct regression proceeds, estrogens are powerless to cause development of these ducts in the caudal segment, where regression has begun. Therefore, varying lengths of oviducts will develop when estrogens are injected, depending upon the age at which the hormones are administered. As a general rule, allowing for individual variations, the later the injection after the 4th day, the shorter the anterior persisting segment. Or, in other words, the older the male embryo at treatment, the greater the distance between the cloaca and the persisting portion of the Müllerian duct. This idea is brought out in Figure 15, where the lengths of oviducts of embryos of from 4 to 18 days' incubation are represented. A complete sum-

mary of the effect of hexestrol upon the Müllerian ducts of males is found in Table 7.

Group III.—Failure to achieve complete inversion of male gonads with the dosage employed in Group II led to the attempt to accomplish this end with higher dosages of hexestrol. Accordingly, 3.75 mg. of hexestrol were injected in each of sixty eggs. On the 4th day 1.25 mg. was administered, followed by 2.50 on the 6th day. Candling showed that the embryos succumbed very rapidly. By 2 days after the second injection, all were dead but one, and this one died on the 4th day.

2. *Females: Group IV.*—This group contains all the hexestrol-treated females, totaling forty-nine in number. In general, the estrogens did not appear to exert any marked effect upon the gonads, but some unusual conditions of both Müllerian ducts and ovaries may be recorded.

The abnormal gonad structure manifested itself as a very thin left ovary, with decided finger-like projections extending from the lateral and caudal margins. This abnormality in some cases was limited to the posterior half of the ovary. Seven of the forty-nine, or one-seventh of the number, showed this trait.

Sectioning and study of these unusual ovaries revealed interesting situations. The case illustrated in Figure 14 and another embryo of the same age and treatment, dosage 2.50 mg. hexestrol, which gave the same general macroscopic picture, were shown microscopically to have atypical left ovaries. These exhibited a cortical region composed largely of cells with flattened nuclei, and only a few cells with typically rounded nuclei were occasionally interspersed. Dantchakoff (1936) labels similar-appearing cortex in the chick, where all the nuclei are flattened, as "sterile." The Progynon-treated female, dosage 0.50 mg., with missing left Müllerian duct, mentioned below (right has small posterior cyst), showed a high degree of "sterility" also. Strangely enough, the female among these Progynon-treated birds that had the best cortex on the right rudiment had a left ovary largely "sterile" and the anterior end of the left oviduct missing. The best-developed cortex on the right rudiment seen anywhere (Case 21H1425b) was treated with hexestrol (2.50 mg.), and in this instance the posterior end of the left oviduct was missing. The right was entire. The left ovary in this case was not "sterile" but smaller than usual, with good cortical development. The right ovary was as well organized into cortex and medulla as any left ovary, and the cortex was as thick as the average, with many cells in synizesis. In other words, it was a typical ovary but of small size. Another hexestrol-treated (2.50 mg.) female, which had a narrow right ovary, mostly cortex of normal thickness, had the posterior end of the left oviduct absent, no right duct, and a left ovary largely "sterile." A final case with deficiency of the left Müllerian duct at the anterior end (right had small posterior cyst) was treated with Progynon (0.50 mg.). Here the right cortex was of normal thickness, and the left, although not "sterile," was retarded in development.

In hexestrol-treated embryos the left Müllerian duct may (1) be totally absent, (2) have varying lengths of its anterior half absent, or (3) have varying lengths of its posterior half missing. The right duct showed these variations, too; this was not so surprising, since it does not persist in its entirety in the untreated female but normally disappears in an anteroposterior direction and seldom, if ever, shows a well-developed cranial

end to the exclusion of the caudal. Figure 14 pictures one of these unusual cases of duct development in the female, which is much more like the male condition treated at a later age. A few similar instances were noted with the higher dosages of Progynon. A summary of the effects of hexestrol upon the Müllerian ducts of females is given in Table 8.

It was stated previously that the right rudimentary ovary in female duck embryos is rather large, as compared with the right rudiment in the chick. Twenty-one estrogen-treated females were sectioned, and thirteen of these showed cortical development (approximately 62 per cent). Some of these displayed only a few lumps, but others had a thick and well-differentiated cortex comparable to the normal, as mentioned above. It must be remembered, however, that these cases were not picked at random but because of an unusually large or small rudiment or some unusual feature of duct development.

D. RESULTS OBTAINED WITH TESTOSTERONE PROPIONATE

The only androgen utilized in this investigation was testosterone propionate.

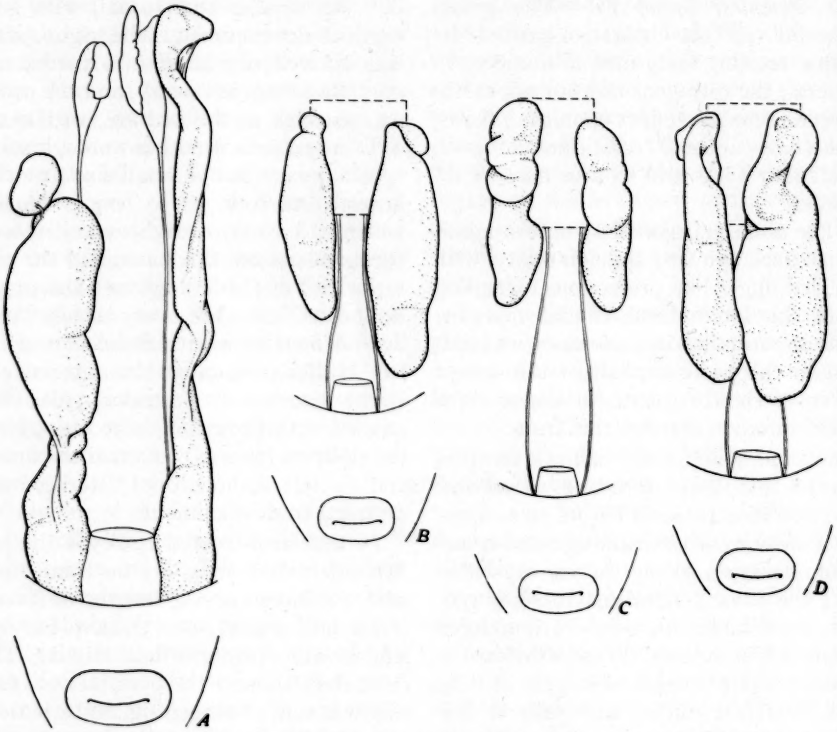

FIG. 15.—Illustrations showing persisting segments of oviducts in males treated with hexestrol, length determined by age of embryo at time of injection. The anterior end is usually the last portion to persist; hence the younger the embryo at time of injection, the longer the segment. Dosage in each instance was 2.50 mg. except *A*, which received 1.25 mg. *A*, injected on 4th and killed on 26th day post-hatch; *B*, injected on 6th and killed on 19th day of incubation; *C* and *D*, injected on 9th and killed on 24th day.

Data on its effects are summarized in Table 9. The dosage was 1.25 mg., and it was administered on the 5th day. The embryos were killed from the 18th to the 26th day. Survival was approximately 30 per cent.

No great deviation from the normal was observed in the eleven surviving females and eight males. The gonads appeared to be normal macroscopically, with possibly one exception in one female, where the cortex was sloughed off in places. In neither sex was there any apparent effect macroscopically upon the Wolffian or the Müllerian ducts.

The gonads of three males and three females were sectioned and examined histologically. Other than the sloughing-off of some cortex in the female (case previously mentioned), no unusual features were observed. Because there were no indications macroscopically of stimulation in the others and since the results obtained from those sectioned were negative, no more embryos were prepared for histological study.

E. RESULTS OF HISTOLOGICAL STUDY OF SEXUAL DUCTS

It has already been mentioned that Müllerian duct hypertrophy was greater

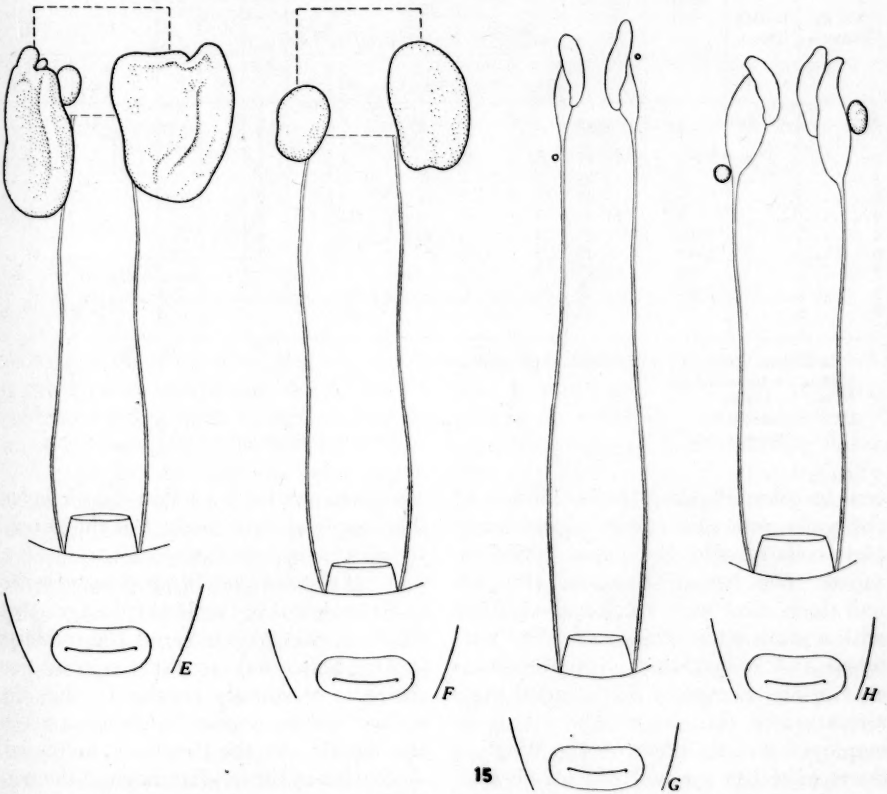

FIG. 15 (*cont.*)—*E*, injected on 12th and killed on 26th day; *F*, injected on 14th and killed on 25th day; *G*, injected on 16th and killed on 2d day post-hatch; *H*, injected on 18th and killed on 27th day of incubation.

in the embryos treated with hexestrol than in those treated with Progynon. In addition to the difference in size, there was a difference in the general macroscopic appearance, which seemed to be occasioned largely by the difference in thickness of their walls. The embryos treated with Progynon possessed ducts that were usually thin-walled, fluid-filled, more or less rounded, and opalescent in color. Because of the thinness of the walls, puncture caused almost complete collapse after the aqueous fluid escaped. The hexestrol-treated embryos had ducts that were thick-walled, filled with a more viscid fluid, somewhat flattened and ruffled, and without opalescent lustre. Escape of fluid caused little alteration of the shape. The estrogens employed had no effect on the Wolffian ducts of either sex, nor did we observe any modifications in Müllerian ducts as a consequence of androgen treatment.

1. *Müllerian ducts in normal females.* —Sections made from a segment of the left Müllerian duct just posterior to the Wolffian body from 26-day females reveal a tube rather oval in outline. The contour of the lumen, however, is not smooth and oval but presents a scalloped effect. The lumen is lined with a layer of columnar epithelial cells, which rest upon a layer of connective tissue that is, in turn, surrounded by a thin muscle layer. The vestigial right oviduct of the normal female was not sectioned.

2. *Müllerian ducts in estrogenized males.* —Histologically, the lower dosages that result in the persistence of the oviducts in the male may produce a condition which is essentially similar to that described for the normal Müllerian duct in the female. As the dosage is increased, distention of the oviduct beyond the normal size because of collected fluids results in a thinning of the oviductal wall, so

TABLE 8

EFFECTS OF HEXESTROL ON MÜLLERIAN DUCTS OF FEMALES

No. of Embryos	Dosage (Mg.)	Injection Age (Days)	Incubation Age (Days)	Left Duct						Right Duct						
				Entire		¾*		½		Entire	¾		½		¼	
				Hyp.†	Nor.‡	A.§	P.‖	A.	P.		A.	P.	A.	P.	A.	P.
4	1.25	4	24–26	3	.....	.....	.....	1	.....	3	.....	.....	1	.....	.....	.....
7	2.50	4	25–26	3	.....	1	1	2	.....	5	.....	.....	.....	.....	.....	2
5	.....	6	19–25	5	.....	.....	.....	.....	.....	5	.....	.....	.....	.....	.....	.....
6	.....	8	25	6	.....	.....	.....	.....	.....	6	.....	.....	.....	.....	.....	.....
2	.....	9	24–25	2	.....	.....	.....	.....	.....	2	.....	.....	.....	.....	.....	.....
4	.....	10	25–28	4	.....	.....	.....	.....	.....	4	.....	.....	.....	.....	.....	.....
9	.....	12	22–2¶	9	.....	.....	.....	.....	.....	7	.....	1	.....	1	.....	.....
7	.....	14	25–4¶	7	.....	.....	.....	.....	.....	.....	.....	.....	.....	7	.....	.....
3	.....	16	2¶	3	.....	.....	.....	.....	.....	.....	.....	.....	.....	2	.....	1
2	.....	18	4¶	.....	2	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2

* The fractions indicate approximate lengths from either end.
† "Hyp." = Hypertrophied.
‡ "Nor." = Normal.
§ "A." = Anterior end.
‖ "P." = Posterior end.
¶ Post-hatching age in days.

that there is a smooth epithelial lining minus folds, inside a very thin muscle layer. In cross-section the lumen was oval shaped and regular in outline, while the lining epithelum was reduced from the tall columnar type to the flat or cuboidal condition, probably because of the pressure of the inclosed fluid. The highest dosages of Progynon produced in one or two instances a thicker wall, occasioned by the evagination of the epithelium into glandlike diverticula, complicating the connective tissue of the wall. A thin muscle layer was added to the outside. The right oviduct in treated males along the margin of the surface bordering the lumen. The contour of the lumen, therefore, was very irregular in outline, as compared with most of those treated with Progynon.

Some males, treated late in the incubation period, developed small globular cysts in the vicinity of the anterior end of the degenerated Müllerian duct. Such were assumed to be persisting portions of these ducts, and sectioning confirmed this assumption.

3. *Müllerian ducts in estrogenized females.*—Only the higher dosages of estrogens caused the persistence of the right

TABLE 9

DATA ON EFFECTS OF TESTOSTERONE

Injection Age* (Days)	Quantity Injected (Mg.)	No. of Eggs Injected	Embryos Killed				Survival (Percentage)
			Age*	No.	Male	Female	
5	1.25	65†	18–26	49	8	11	0.29
Sex ratio (per cent)	.....	.....	.....	.....	43	57	.....

* Incubation days.

† The discrepancy between number of eggs injected and embryos killed is due to the death of the embryos in the interim.

showed all the histological features found in the left and, in addition, was found degenerated into a short vestige, as may be found on the right in the normal female.

A cross-section from the same region as in females shows how tremendously the glandular layer has been augmented following hexestrol treatment (2.50 mg.), as compared with Progynon treatment (1.50 mg.). The walls become exceedingly thick and exceedingly glandular, causing the muscular layer to become reduced in places from a compact layer to a thin, mere network of muscle cells. The columnar epithelium lining the lumen dips deeply toward the muscle layer at intervals, producing an interruption in the continuity of the thick-walled condition

oviduct in its entirety, and for that reason it was found with less frequency among the embryos treated with Progynon than among those receiving hexestrol (see Tables 4 and 5). Histologically, the right duct behaves about the same as the left does in response to varying dosages, and both right and left ducts conform to the description given for estrogenized males.

4. *Wolffian ducts in normal males.*—In view of the marked effect of testosterone upon the mesonephric system of the chick (Dantchakoff, 1937; Willier, Rawles, and Koch, 1938) and the apparent lack of effect in the duck with the amount injected, as determined macroscopically, it seemed desirable to make histological

preparations of the Wolffian ducts. Segments about the length of the gonads were taken just posterior to the Wolffian body and almost immediately anterior to the cloaca and were sectioned. When microscopic examination disclosed no readily observable difference between these sections and those of control embryos, ocular micrometer measurements were made of the lumen and the entire diameter of the Wolffian ducts. Three readings were made from the slide containing serial sections taken from the anterior segment of the duct, and three from the posterior segment. The sections chosen for measurement were from approximately the beginning, the middle, and the end of the slide. These figures were averaged for all the individuals in a particular category and were compared with the averages of controls obtained likewise.

The sections from the anterior end of the duct in the normal individual generally exhibited a lumen more oval than circular; it was lined by a layer of columnar epithelial cells slightly larger than the lumen, with the result that there was some buckling of the epithelial layer into the lumen. Outside the epithelium was a thick wall of connective tissue surrounded by a thinner muscular layer.

Sections from the posterior segment were decidedly more oval in contour, indicating compression. There was a considerable increase in the entire diameter of ducts; the average through the long axis was three times that at the anterior end. The diameter of the lumen at the posterior end was almost four times the anterior lumen diameter. At the posterior level the lumen was very irregular because of infolding of the mucosal lining, and the increase in diameter of the duct was due to the larger lumen, as well as to augmentation in thickness of the muscular wall.

5. *Wolffian ducts in androgenized males.*—Sections of Wolffian ducts were obtained from the anterior and posterior ends in the manner described for normal males and were examined microscopically. Ocular micrometer measurements of the diameter of the lumen and of the entire duct were made. The average reading at the anterior level was a fraction greater in the treated than in the untreated, but at the posterior end the fractional difference was in favor of the normal. These differences tend to support the observations made macroscopically that there appears to be no definite effect produced by testosterone upon the Wolffian ducts under the conditions given.

6. *Wolffian ducts in androgenized females.*—Sections made from the anterior and posterior ends of the Wolffian ducts did not show any indication of stimulation. Measurements of the diameter of the entire duct and of its lumen were made at the two levels above mentioned in both normal and androgenized females, and the readings showed a greater average diameter of the ducts in the untreated females at both ends.

From these data it appears that, on the whole, there has been little or no hypertrophy of Wolffian ducts as a consequence of testosterone treatment with the relatively high dosage administered.

V. DISCUSSION

Detailed investigations of the origin of the germ cells and the structure and development of the sex glands in the chick have been carried out by a number of investigators, notably Swift (1914, 1915, 1916), Firket (1914), D'Hollander (1904), Semon (1887), and Hoffman (1892); but only Laulanié (1886) mentions a homologue of the ovarian cortex, which he refers to as a *couche ovigène* that may be observed on the left testis be-

tween the 7th and the 12th days of incubation. In referring to this work, Swift (1916) states: "According to his idea, the gonad was not indifferent but hermaphroditic. Later on in development one of the sexual elements degenerated and the gland became male or female as the case might be. This idea is of interest only because of its novelty and historic interest." Swift's detailed description of the embryonic development of the testes fails to include any mention of a persisting thickened germinal epithelium on the left testis. Concerning the epithelium, he states that by the end of the 9th day of incubation the germinal epithelium is reduced to a single layer of cuboidal cells.

Since these observations were made by Swift, it has been demonstrated that Laulanié was correct in postulating a bisexual condition obtaining in the embryonic left testis of the chick (Dantchakoff, 1936; Willier *et al.*, 1935, 1936, 1937; Wolff and Ginglinger, 1936*a*); and representatives from the other two classes of Amniotes—Reptilia and Mammalia—as well as additional Aves, have been shown to possess bisexual gonads bilaterally or sinistrally. Among reptiles, Risley (1933) demonstrated bisexuality in the musk turtle, Forbes (1938) in the alligator, Dantchakoff (1938) in the lizard; among birds other than the chick, Burwell (1931) in the duck, Witschi (1935) in the sparrow; and among mammals, Hett (1932) in the cat, dog, squirrel, mouse, rat, guinea pig, sheep, calf, pig, and man.

In normal development the bisexual components of the testis experience various fates. According to Risley (1933): "Both testes and ovaries develop out of a bisexual primordium, and the bisexuality of the early gonads furnishes a morphological foundation for the eventual formation of either sex." In the male turtle the distinct ovarian cortex which is formed during embryonic development gradually retrogresses. Witschi (1935) reports a similar condition to that in the musk turtle as obtaining in the common sparrow. The male chick possesses this potential cortex in the form of a thickened persisting germinal epithelium "from at least the seventh to the eleventh day," after which it undergoes retrogression (Willier, Gallagher, and Koch, 1937).

Grünwald (1934*a*, *b*) gives an account of the normal development of the sexual cords in several mammals, namely, the rat, mouse, pig, and man; and in the latter he describes fully the development of structures which he designates as secondary sexual cords that are analogous to the cords of second proliferation in the female; therefore, he calls them the "secondary sexual cords of the male." He states that no one had given an account of the development of these cords in the male, as far as he was able to ascertain in his survey of the literature. He mentions Hett's (1932) observation of a persisting germinal epithelium in certain mammals, but Hett did not record the development of this epithelium into the secondary sexual cords.

According to Grünwald's (1934*b*) account, there develops on both testes of the human embryo, outside or within the tunica albuginea, sexual cords that differ from the primary cords by the size and staining affinities of the cells. As these cords develop, the tunica, which separates the primary and secondary cords, disappears; and a new tunica is formed outside the secondary cords, resulting in the intimate contact of the two sets of sexual cords. Not only do these cords have contiguity, but union is established between them, and within a short period it is impossible to distinguish primary

from secondary cords. These secondary cords are regarded as homologous to the cords of second proliferation in the female, but they do not arise at a fixed time as in the female, neither were they found in all the embryos examined. At the time, Grünwald was not sure whether they were present in all embryos or whether they had escaped his notice because he did not examine the gonads at the propitious moment for their observation, since he states that their time of development is not predictable as in the ovary and that they, soon after their formation, blend indistinguishably with the primary sex cords and all become incorporated within the same tunica. Hence he concludes that fundamentally there is no difference between the human ovary and testis with reference to the origin of the sexual cords. The actual difference is in the manner of participation of the two sets of cords in the formation of the gonads as a whole; there are fewer secondary cords involved in the architecture of the testis than in that of the ovary.

The observations in our investigation upon the normal development of the left testis of the duck appear to have much in common with those of Grünwald upon the human testis. In the duck, which follows the asymmetrical gonad development found in most birds (Domm. 1927), the formation of secondary sexual cords is limited chiefly to the left testis, with only an occasional feeble attempt on the right; whereas, in man, secondary sexual cords are found in both gonads (Grünwald, 1934*b*). These secondary cords in the duck increase in size by proliferation and division of germ cells, located in the persisting germinal epithelium found on the left testis (Burwell, 1931), so that this layer becomes characterized by lobulations, as observed for cortical-cord formation in the chick (D'Hollander, 1904; Firket, 1914; Swift, 1915); however, these lumps of cells in the duck may cause surface elevations, as well as protrusions inwardly, which encroach upon the tunica albuginea. Continued proliferation of cells in these lobulations results in the production of definite cords, which appear analogous to the secondary sexual cords shown in Grünwald's (1934*b*) illustrations of the human embryonic testis. These cords in the duck also are readily distinguishable from the primary cords by a difference in affinity for certain stains. They form a conspicuous investing outer layer on the gonad. The layer is always thicker at the posterior end of the gonad and in some cases is lacking entirely at the anterior end. The final disposition of this layer of cords was not determined with certainty; however, there is some evidence that they may be incorporated in the testicular structure and become indistinguishable from the cords of first proliferation, as postulated by Grünwald for the human embryo.

Studies on the structural composition of the ovary of birds, as well as of mammals and certain other vertebrates, have clearly shown a bisexual organization. The primary sex cords which constitute the medulla are morphologically equivalent to the sex cords of the testis, while the cortex formed from the secondary sex cords is distinctly ovarian. Analysis of the freemartin condition (Lillie, 1917) demonstrated that the medulla is not only the morphological homologue of the testis but is also potentially a testis. A similar condition prevails in birds, where the right rudimentary gonad, composed chiefly of medullary tissue, develops into a testis, following sinistral ovariotomy (Benoit, 1923, 1924, 1926; Domm, 1924,

1927, 1929, 1931; and others). In each case the first set of sexual cords differentiates into seminiferous tubules.

The bisexual organization of the ovary is not peculiar to birds and mammals but appears to be demonstrated also for Amphibia, as shown by the studies of Witschi (1929, 1934), Burns (1935), and Humphrey (1935), as well as for reptiles, as revealed by the observations of Risley (1933) in the musk turtle and Forbes (1938) in the alligator. It is therefore clearly demonstrated that the ovary of many, if not all, vertebrates consists morphologically of a rudimentary testis, the medulla, surrounded by the specifically female cortex, and that, as has been equally well shown, this morphological composition is an expression of corresponding physiological capacities. Hence the potentialities for sex inversion in the female were demonstrated long before this condition was known in the male.

The first attempts to duplicate the freemartin condition were undertaken in the form of grafting experiments. Minoura (1921), who pioneered in this work on the chick, claimed to have obtained sex reversal by gonad transplants; but Greenwood (1925), Kemp (1927), and Willier and Yuh (1928) did not agree with the findings of Minoura. Willier and Yuh demonstrated that gonad grafts were nonspecific in producing sex reversal and that results similar to Minoura's could be induced by certain conditions of incubation, such as low temperature, and by various operative procedures, as well as by grafts of nongonad tissue. So the expedient of grafting, which was successful in bringing about sex reversal in Amphibia (Witschi, 1927; Humphrey, 1929; Burns, 1931), was a failure in so far as the chick was concerned.

With the isolation and purification of sex hormones and their ready availability in pure form, this problem was reinvestigated with more fruitful results. Kozelka and Gallagher (1934) injected estrogens into hen's eggs; and, following their success in inducing the left gonad of the male to develop into an ovotestis, subsequent investigators have demonstrated complete transformation of the left testis into an ovary in the chick embryo. Willier *et al.* (1937), using sex-linked chicks, observed that theelin and theelol, in dosages of 1.0 and 2.0 mg. injected into the albumen, caused complete inversion of the left testis into an ovary and the right testis to become reduced in size and flattened and to develop ovarian medullary tissue, though its testicular character was never completely lost. Wolff and Ginglinger (1935*a*) found that approximately 100 I.U. of folliculine, when applied directly to the blastodisk, would produce an intersexual condition in all male embryos and that 500 I.U. or over were required to bring about complete reversal. Dantchakoff (1936), by the introduction of 2,500 mouse units of folliculine into the allantoic sac, stated that she was able to bring about complete sex reversal in the male chick at will.

In the present investigation no case of complete reversal of the left testis of the duck was observed, despite the fact that dosage was stepped up to a point (3.75 mg. of hexestrol) at which all embryos of sixty eggs injected were dead by the 10th day of incubation. The initial dosage was 0.25 mg. of Progynon, and this was gradually increased to 1.5 mg. in different series of embryos. There was some indication that the degree of cortical differentiation in duck embryos is conditioned by the increase in estrogen administered,

as found by Wolff and Ginglinger (1935*b*) and by Willier *et al.* (1937) in chick embryos; but the highest dosage of Progynon administered did not cause the formation of a thick, well-differentiated cortex, comparable to that in the normal female. However, in some of the treated cases receiving the maximum dosage of Progynon a well-developed surface layer was found as thick as the normal cortex in females and resembling that layer before synizesis sets in. The striking feature here, as well as in some other instances noted in this study, was the apparent method by which this "cortex" was being formed. Underlying testicular cords appeared quite obviously to be growing out to the periphery of the gonad, where, by continued development and eventual severance from the underlying cords, they formed or contributed to the "cortical" layer investing the left gonad. Considerable skepticism prevailed as to the validity of applying the term "cortex" to the layer arising in the manner described, particularly in the absence of cells in synizesis, until cases were found in which the gonad showed, all in the same embryo, (1) instances of connecting testicular cords with this outer layer, (2) an area in which this layer was completely detached from the underlying cords, and (3) a patch of fully differentiated cortex.

In reviewing the literature, no reference was found that mentioned such a method of cortex formation. However, it does seem to be in harmony with the principles of development as set forth by Weiss (1939, pp. 320–30) with reference to determination in organ rudiments. Cortex, formed in this manner, may require a somewhat longer period to differentiate fully than when formed from the germinal epithelium, which would account for the absence of cells in synizesis in many of the cases observed. It should, of course, be emphasized that, in many embryos, cortex was also observed to be developing from the thickened persisting germinal epithelium on the left testis, as observed by others.

The relation between dosage and degree of cortical differentiation was clearly indicated in these experiments. When Progynon failed to cause complete inversion of the testis, diethyl stilbestrol and hexestrol, which were available in higher concentrations, were tried. A very high degree of intersexuality was obtained with the use of these estrogens. The gonads revealed cortex along the posterior portion as thick as that of control females of comparable age. Usually, cortical hypertrophy was more pronounced at the posterior end, with a tendency to become thinner as the anterior end was approached; but cells in synizesis appeared first at the anterior end. This differential in thickening is in keeping with the condition of the persisting epithelium in control males. The difference in degree of response of the duck gonad to Progynon, stilbestrol, or hexestrol is not regarded as attributable to any inherent differences in the action of the hormones but rather to a difference in the dosages used, although we have no data on relative potencies.

In spite of the fact that excellent cortex was obtained following the use of hexestrol, the medulla was apparently qualitatively unchanged as a result of treatment, regardless of the estrogen employed. Forbes (1938), using Progynon on sexually immature alligators, noted that this hormone exercised an effect similar to that observed in these experiments, namely, that there is pronounced cortical hypertrophy but no noticeable effect upon the medulla qualitatively. In addition, he asserts that

there is no significant quantitative change in the medulla, and he interprets this stability as evidence that the estrogen acts by direct stimulation of the cortex instead of by inhibition of the medulla. However, it has not been established indubitably in the duck that there is no volume change in the medullary component of the gonad following estrogen administration, as Forbes claims for the alligator. In the majority of the estrogen-treated males it appears rather certain that there has been no decrease in volume; but in some of the males receiving the highest dosages the difference in total gonad size in favor of the left was immediately evident; however, this disparity could be due to the addition of cortex on the left as well as to reduction of medulla on the right or both. In both testes the medullary component was usually well developed and clearly testicular in type. In Forbes's experiments, cortical differentiation occurred on both testes instead of on the left testis only.

The right gonad in estrogen-treated males usually appeared broader macroscopically than the left, and the latter was, as a rule, longer. In a few of the embryos receiving the highest dosage the right gonad was noticeably smaller than the left, which, as stated above, could be due to the added cortex on the left or to reduction of testicular cord volume or both. Microscopically, the right testis of estrogenized males occasionally showed a few cortical lumps. These were never observed to attain any appreciable thickness, and the frequency with which they were encountered seemed to coincide with the number of cases showing persisting patches of thickened epithelium in controls. There appeared to be no modification of testicular cords, nor could we be certain of a decrease in volume. Willier *et al.* (1937) report no striking change in the right gonad of estrogenized male chick embryos except in those cases showing extreme modification of the left, in which case the right becomes reduced in size, flattened, and contains medullary tissue. This differs from the condition found in the duck, where the right exhibits a flattening with no medullary tissue, when the left attains merely an intersexual condition.

Several investigators cite evidence to show that estrogens may stimulate cortical development in the female. Dantchakoff (1935*b*) states that Progynon in the female chick acts like a whiplash in accelerating histogenetic differentiation. Forbes (1938) records hypertrophy of the ovarian cortex of both ovaries in immature female alligators as a result of estrogen treatment. The increase in volume may be as much as 90 per cent.

Macroscopic and histologic study of estrogen-treated female duck embryos reveals an apparent hypertrophy of cortex on the left ovary in some instances, as compared with controls. Too, it was only in estrogen-treated females that a cortex of normal thickness with cells in synizesis was found in the right ovary. It would seem that estrogens exert a specific effect upon the development of cortex in the duck embryo. These results would appear to contradict the observations of Moore and Price (1932) that gonad hormones have no direct effect on the gonads of either the same or the opposite sex; however, it should be noted that their observations were based on experiments carried out upon adult rats, animals with fully developed, mature, and not embryonic reproductive systems. The results obtained in another class of vertebrates where adult or embryonic undifferentiated gonads are subjected to administered sex hormones may, however, not be comparable. It should be noted that

Willier *et al.* (1935, 1936, 1937) observed that in zygotically determined females the right and left ovaries remain usually quite normal in size, form, and histology, regardless of dosage of estrogens administered, whereas in genetic males the testes were reduced in size, though essentially normal in form and structure following administration of testosterone propionate.

The estrogens employed in these experiments apparently have no specific effect upon the Wolffian ducts, but they do have very specific effects upon the Müllerian ducts. This is in keeping with the observations of Willier *et al.* (1935, 1936, 1937), Wolff and Ginglinger (1935*a*), and Dantchakoff (1936). The fact that greater hypertrophy was obtained with stilbestrol and hexestrol than with Progynon doubtless results from the fact that higher concentrations of the latter were administered. In addition to greater volume, the hexestrol-treated ducts had thicker walls correlated closely with a more abundant glandular development. The degree of hypertrophy of Müllerian ducts was not always an index of the extent of cortical development, for the ducts might show good development and the gonads little or no cortex. Similar observations were made by Willier *et al.* (1937) in the chick. These authors observed that male chicks with completely reversed left gonads had oviducts strikingly similar to those of the normal female. This is in contrast to the modification found in the duck, where low dosage produced almost typical female Müllerian ducts but scant cortical stimulation, whereas the highest dosages, which caused greatest cortical development, occasioned monstrously overdeveloped ducts on both sides.

Willier *et al.* (1937) and Forbes (1938) have observed in the chick and alligator, respectively, that the most persistent portion of the Müllerian duct in the estrogenized male is the anterior end. This harmonizes with observations in the duck and is in keeping with what might be expected from the manner of normal degeneration of these ducts—from the caudal to the cranial end. Furthermore, in the duck the length of the persistent portions is related to the age at which the embryos receive the hormone. In other words, the earlier the injection, the more nearly normal length the oviduct is likely to be.

The failure of testosterone propionate to exercise any appreciable effect upon the female or male urinogenital system in duck embryos is in contrast to the conclusions of Willier *et al.* (1935, 1936, 1937) with respect to the action of bull-testis extracts upon the chick. Dantchakoff (1937) also observed that testosterone caused an enlargement of the Wolffian ducts in the chick and that, while it was depressing to the cortex and Müllerian ducts and stimulating to the mesonephric tissue, reversal of sex from female to male was not accomplished. She tried at various times to bring about transformation of females to males, having used X-ray irradiation before androgen treatment, which resulted in partial inversion of females (1935*b*).

Willier, Rawles, and Koch (1938) made an exhaustive study of the effects of male hormones upon the genitourinary system of chick embryos. Here, an effect is noted for bull-testis preparation, which causes hypertrophy of the Wolffian ducts in male and female. Willier *et al.* (1938) also described a masculinizing action of dehydroandrosterone, androstenedione, and testosterone propionate upon genetic females with enormous hypertrophy of the Wolffian ducts.

Wolff (1935*a*, *b*, 1936*a*) recorded a

dual action of androsterone upon chick embryos by which males are feminized and females masculinized; and Wolff and Ginglinger (1936*b*) demonstrated that certain androgens have an estrogen-like action upon immature female mice, as well as a masculinizing action on combs of capons. Testosterone, which did not show feminizing effects in the experiments of Willier *et al.* (1938) or in the experiments here reported, is included among the androgens mentioned by Wolff and Ginglinger as possessing certain estrogen-like qualities. The possibility that testosterone might possess these qualities was not ruled out by Willier *et al.* (1938) in the light of the observations of Wolff and Wolff (1936), who noted that in two breeds of chickens—the Leghorns, on the one hand, and a cross between Light Sussex females and Rhode Island Red males, on the other—there was a difference in the amount of androgen required to effect the intersexual condition—hence testosterone might cause both masculinization and feminization in other breeds.

The references on work with androgens just cited show how varied the results may be, depending upon the androgen, the experimental animals, and other conditions. However, all seem to agree upon the point that inversion of females is more difficult to bring about in the embryonic stage than inversion of males. This fact, along with the duck's demonstrated resistance to estrogens, may account in part for failure to obtain any demonstrable results with testosterone.

Because of the evident effect of testosterone upon the gonads and Wolffian ducts of the chick as reported by other investigators and because of the apparent absence of an effect observable macroscopically in the duck, histological study of these structures was made. Our study did not give conclusive proof that this androgen caused any modification in these structures. It is possible, however, that a higher dosage of testosterone propionate than that administered in our experiments might bring about a modification of sexual characters in the duck, since it has proved more resistant, in general, to the hormones administered than the chick, where Willier *et al.* (1938) found that much less than 1.0 mg. of estrogen was sufficient to feminize males completely, while 2 mg. or more of androgen only partially masculinized females. In a recent paper (Moore, 1944) the problem of variations in the effects of hormones and conflicting results obtained by treatment with sex hormones in the various classes of vertebrates is discussed in some detail.

Wolff and Ginglinger (1936*a*) state that hormones have no effect upon the gonad of the chick if injected after the 8th day of incubation and that only the cortex is modifiable this late in incubation, the medulla having earlier ceased to be responsive. Dantchakoff (1935*b*) observed that embryos injected on the 4th day showed the greatest modifiability, those injected on the 6th, 7th, and 8th days were intermediate in response, while those injected on the 10th day showed very little effect. Willier *et al.* (1937) link the effective period of hormone action with the interval beginning with sex differentiation, namely, about the 6th day, and terminating with the retrogression of the incipient cortex, about the 11th day.

In the case of the duck, the best cortical development was obtained in embryos injected on the 4th day. Those injected on the 6th day were highly modified but not so fully; however, there were fewer cases in this category. Embryos injected on the 8th day showed a definite falling-off in cortical stimulation, on the

9th day a further drop was evident, and after the 10th day there was no evidence of cortex formation from the germinal epithelium.

According to Wolff (1936*b*) the Müllerian ducts persist and develop in the male chick if injection occurs before the 10th day. Willier *et al.* (1937) suggest that oviduct response to hormones probably ceases after the 11th day, when retrogression is complete.

Our study of normal development in the duck discloses that the Müllerian ducts complete their retrogression about the 18th day, and it appears that hormone treatment after that date is ineffective in causing hypertrophy of these structures. The whole duct does not show development in the male following later injections, but a segment of the anterior portion, which represents the last part to degenerate, may.

The oviduct in the duck, therefore, seems to agree with that in the chick with reference to the period of modifiability. This period is longer in the duck by 1 week, which appears to coincide with the longer period of incubation. This observation on the oviduct would indicate that the gonad might also be modifiable a week longer in the duck than in the chick. In actuality, the effective period here is not a week longer in keeping with the longer period of embryonic development but agrees with observations on the chick (Dantchakoff, 1935*b*), namely, from the 4th to the 10th days of incubation. Estrogen treatment after the 10th day causes, in a few cases observed, some testicular cords to push through to the surface, forming large lumps, which resemble those mentioned previously in connection with cortex formation. Embryos injected as late as the 14th day showed this condition. In some individuals injected at earlier stages, testicular cords were also observed to push to the surface and form a layer in this manner, which showed typical cortical differentiation. Since these cords can be induced by estrogen treatment at 14 days to form this outer layer, it is possible that the layer might differentiate into cortex if allowed to develop a sufficient period. It may be expected that cortical formation of this type tapers off with increase in age at the time of injection, just as it does in cortical development from the germinal epithelium. In other words, a stage is reached when irrevocable determination has been attained by the testicular cords. The time at which this occurs has not been accurately determined, but after the 14th day there was little indication of formation of a surface layer from testicular cords in the manner described.

These observations show that the primary sexual cords may under suitable conditions undergo inversion and form cortical cords and theoretically would indicate that the indifferent gonad, which consists of a primary set of sexual cords, should be capable of transforming in either direction.

VI. SUMMARY

1. The effects of several estrogens and an androgen upon the development of the gonads and gonoducts of the White Pekin duck were studied following injection of these hormones into the incubating egg. As a basis for comparison, normal development was studied beginning from the 9th day of incubation, soon after sex differentiation, to 24 days after hatching.

2. The germinal epithelium of the left testis does not degenerate soon after sex differentiation, as claimed for the chick, but proliferates additional cords, beginning about the 14th day, which become

incorporated into the testis along with the primary cords. The epithelium is found best developed at the posterior end of the gonad. A well-developed germinal epithelium was never noted on the right testis.

3. The Müllerian ducts in the male degenerate posteroanteriorly and have disappeared by about the 18th day. The right oviduct in females degenerates anteroposteriorly, and at 18 days only a posterior vestigial tip remains.

4. The estrogens—alpha-estradiol benzoate (Progynon-B), diethylstilbestrol, and hexestrol—and the androgen—testosterone propionate—were employed. The amount of alpha-estradiol administered varied from 0.25 to 1.50 mg., hexestrol varied from 1.25 to 3.75 mg., and stilbestrol and testosterone were given only in 1.25-mg. quantities.

5. Injections were made from the 3d to the 18th day of incubation, and embryos were killed at from 9 days' incubation to 24 days' post-hatching.

6. Administration of 0.25 mg. of alpha-estradiol in some cases caused the development of a thin cortical layer on the left testis. Doubling the dosage produced slight improvement in cortical development. The highest dosage of alpha-estradiol, 1.50 mg., failed to bring about complete inversion of testes, although cortical development was more pronounced. The right testis was slightly smaller in some of the cases but distinctly testicular, regardless of dosage.

7. Injection of 1.25 mg. of stilbestrol brought about a pronounced intersexual condition in the left testis.

8. Dosages of 1.25–2.50 mg. of hexestrol produced the greatest degree of cortical differentiation observed in males. The cortex at the caudal end of the left testis was as thick as that of the normal female and showed many cells in synizesis. The medullary portion of the gonad retained its testicular character. The testicular cords of the right testis showed no modification due to hexestrol treatment. This gonad as a rule was smaller in size.

9. Injection of 3.75 mg. of hexestrol on the 4th–6th days caused death of all embryos by the 10th day of incubation.

10. No definite effect upon the ovaries was noted in any of the estrogen-treated cases.

11. A 1.25-mg. dose of testosterone propionate apparently had no effect upon the gonads or Wolffian ducts of either sex.

12. The lowest dosage of alpha-estradiol usually produced oviducts in males like those of the normal female. The highest dosage caused both oviducts to become greatly hypertrophied. Both oviducts in females were greatly enlarged following injection of the higher dosages of alpha-estradial, the right persisting in its entirety when injection occurred as late as the 12th day.

13. Treatment with 1.25 mg. of stilbestrol produced considerable hypertrophy of Müllerian ducts in four males and four females, the only survivors of forty-five embryos at 22 days. The high degree of toxicity of this hormone discouraged its further use.

14. With hexestrol treatment in males both Müllerian ducts were usually fully developed when injection occurred on the 4th day and they became so huge as to be grotesque in appearance. The walls were thick and exceedingly glandular. The later in incubation the estrogens were administered, the shorter became the persisting segments of oviduct. These occurred at the anterior end. Oviducts were not usually found when injections were made after the 18th day. Müllerian

ducts in females revealed the same degree of modification as in the male. There was no evident effect of estrogens upon the Wolffian ducts.

15. The greatest degree of reversal was obtained in embryos injected on the 4th day. Those treated on the 5th and 6th days were almost as good, but there was a definite falling-off in degree of cortical development from the 8th to the 10th day. After the 10th day no cortical differentiation was observed to occur from the germinal epithelium; however, underlying testicular cords were seen pushing through to form a surface layer in embryos injected as late as the 14th day, and in some instances these cords appeared to be differentiating into cortex.

16. Cortical cords may therefore form from the germinal epithelium of the left testis or from primary testicular cords, which have grown out and formed a layer on the surface of the gonad.

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PLATE I

(Initial magnifications reduced by one-half for publication.)

FIG. 16.—Normal left ovary of 22-day embryo. Note cells in synizesis in middle portion and its absence in lateral margins. Initial ×120.

FIG. 17.—Showing enlarged section of cortex from central area of Fig. 16. Initial ×450.

FIG. 18.—Normal left testis of 14-day embryo, showing early formation of cords on surface. Initial ×120.

FIG. 19.—Normal left testis of 18-day embryo. Note continuous layer of cords on surface of gonad. Initial ×120.

FIG. 20.—Normal left testis of 24-day embryo, showing maximum thickening of cords located near posterior end. Initial ×120.

FIG. 21.—Same as Fig. 20 but near middle of testis. Initial ×120.

FIG. 22.—Normal left testis of 28-day embryo, showing group of newly formed cords inside tunica albuginea in close contact with primary cords. Initial ×120.

FIG. 23.—Early stage in formation of cortex from germinal epithelium of left testis. Embryo received 0.50 mg. of Progynon on 4th day and was killed on 18th day. Note underlying testicular cords with inclosed primordial germ cells. Initial ×120.

FIG. 24.—Illustrating differentiation of cortex from germinal epithelium of left testis with lowest dose of Progynon (0. 25 mg.). Embryo was injected on 3d day. Age 25 days. Note typical testicular cords underlying cortical layer. Initial ×90.

PLATE II

(Initial magnifications reduced by one-half for publication.)

FIG. 25.—Illustrates the maximum development of cortex observed. This section was taken from the left gonad of the embryo shown in Fig. 13. It received 1.25 mg. of hexestrol on the 4th day. Age 26 days. Note testicular character of medullary area underlying cortical layer. Initial ×120.

FIG. 26.—Higher magnification of a portion of cortical layer shown in Fig. 25. Initial ×180.

FIG. 27.—From left gonad in Fig. 25, showing relation between surface cortex and underlying testicular cords. Initial ×120.

FIGS. 28, 29, and 30.—These sections are from the same embryo and reveal stages in the formation of differentiated cortex from primary cords of the left testis. Fig. 28, outgrowing cords; Fig. 29, surface layer cut off from the underlying tubules; and Fig. 30, patch of dif-

PLATE I

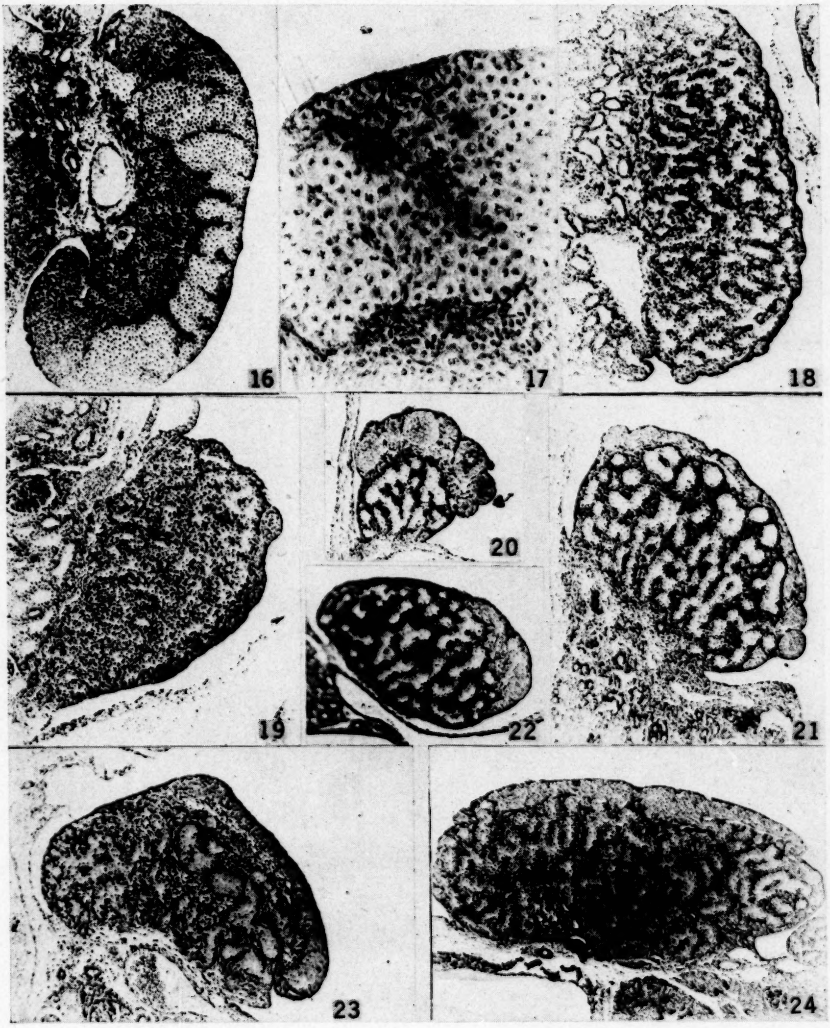
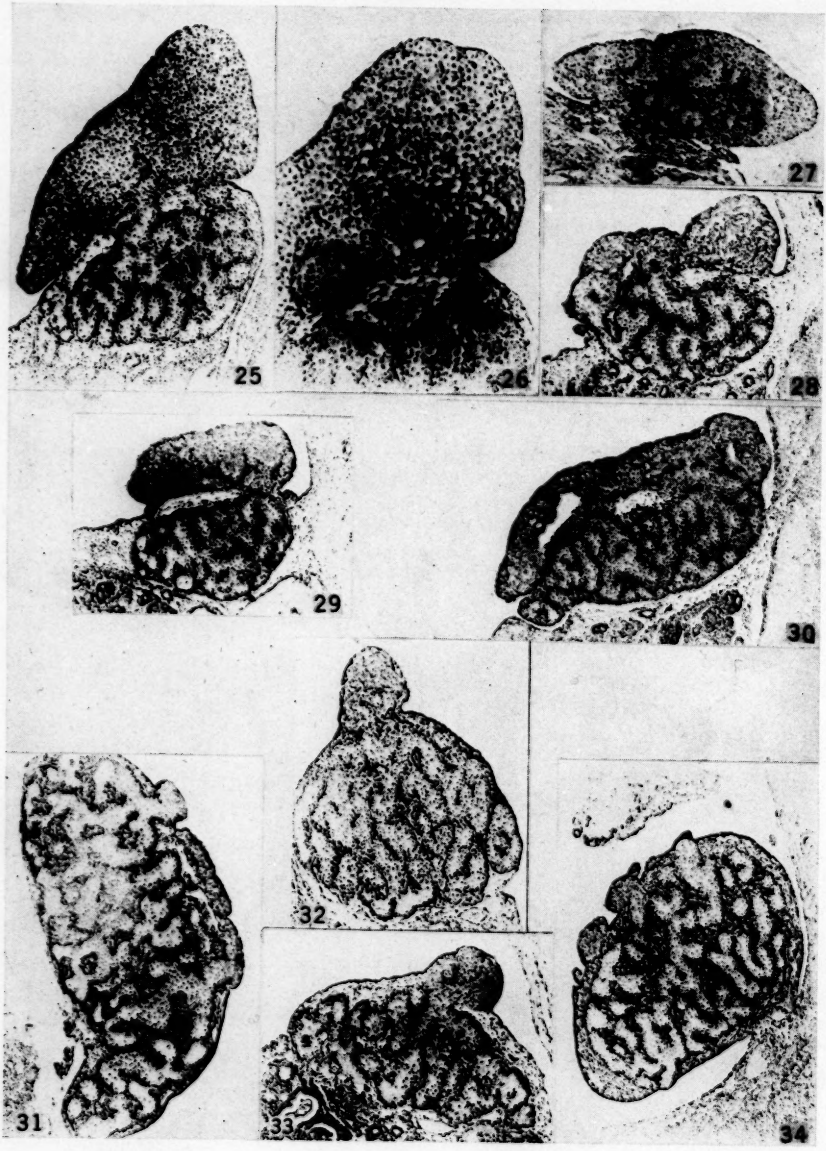


PLATE II



ferentiated cortical cells in the surface layer. The embryo received 1.0 mg. of Progynon on the 6th day and was killed when 26 days old. Initial ×120.

FIG. 31.—Illustrates an early stage in outgrowth of testicular cords forming surface lumps. Embryo received 0.25 mg. of Progynon on 4th day and was killed on 22d day. Initial ×120.

FIG. 32.—Shows tubule of left testis connected with surface cap of cells. This layer becomes differentiated into cortex toward the anterior end of gonad. Embryo received 0.50 mg. of Progynon on 4th day and was killed on 25th day. Initial ×120.

FIG. 33.—Shows outgrowth of primary cords to surface, where differentiation follows. This embryo received 1.0 mg. of Progynon on 6th day. Age 22 days. Initial ×120.

FIG. 34.—Testicular cords of left gonad pushing to surface, following injection of 2.50 mg. hexestrol on 14th day. Embryo killed on the 28th day. Initial ×120.

NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

THE INDIVIDUAL IN SIMPLER FORMS,[1] BY ARTHUR RUSSELL MOORE

Reviewed by LIBBIE H. HYMAN
American Museum of Natural History

THIS is an interesting book dealing with certain selected types of coordinating mechanisms, especially the nervous system. The author begins with a brief consideration of the vitalism-versus-mechanism question; but his definition of vitalism as the "impossibility of understanding life in terms other than those of life" (p. 1) appears to the reviewer to be a definition of organicism and not of vitalism as usually understood. As the author admits (p. 2) that in biological matters the whole is usually more than the sum of its parts, he classifies as an organicist and not as the mechanist he professes himself.

The first chapter deals with the properties of myxomycete plasmodium, on which the author has made substantial contributions. This is cited as an organism of which the whole is equal to the sum of the parts, in that any part (if not too small!) exhibits all the properties of the whole. Stress is laid on the importance of the physicochemical structure of the protoplasm, especially the presence of micelles, in the behavior of the plasmodium. But the analysis fails to reveal the cause of the streaming or the factors operative in initiating the streaming.

The next two chapters discuss early embryonic processes, especially the formation of the blastula and gastrula. Apparently, it is to these chapters that the author refers when he states (p. 3) that his essential purpose is the demonstration that "the emergence of new patterns of form and of behavior is made possible by a third factor, namely, the spatial arrangement of the morphological elements." The phrase "morphological elements" is ambiguous, but it would appear that thereby the author means "cells"; but, in fact, the role of spatial arrangement of cells in form and behavior is considered only in regard to the blastula. Here the task is defined as the description of "the means by which a multitude of cells, parts of a previously existing undifferentiated whole, becomes organized." The author knows perfectly well, as numerous statements indicate, that the egg is not an undifferentiated whole; and, in fact, the organization of the egg is invoked to explain the organization of blastula and gastrula. In regard to the blastula, the discussion centers about cohesive and osmotic forces causing the cells to adhere and the means whereby the blastula develops an internal medium more or less independent of the external one. But nothing is said of the fact that the blastula is polarized

[1] Eugene, Ore.: University of Oregon Press, 1945. Pp. x+143. $1.25.

and has the properties of an axiate organism. In regard to the gastrula, the author follows the usual argument that archenteron formation is a necessary and inevitable step because the organism must feed. The reviewer has elsewhere shown the fallacy of this argument in that many animals have solid gastrulae and that organisms of even considerable size can nourish themselves by means of intracellular digestion. There are even animals of what the author calls the "ganglionic level" of structure that have no digestive lumen and take food organisms of considerable size into an interior mass of cells, i.e., the flatworm group Acoela. Consideration of the gastrula is devoted chiefly to attempts to explain on mechanical grounds the process of invagination, but without success. These two chapters, rather than supporting the mechanistic point of view, as the author supposes, illustrate, instead, the futility of the standard mechanistic approach when dealing with such problems as individuality and organization. These essential matters are sidetracked for discussions of physicochemical properties that, although interesting and valuable in themselves, throw but little light on the organization of the embryo as a whole.

In the following three chapters the author is on firmer ground and gives a good account of the functioning of the nervous system in the behavior of animals selected as illustrating three grades of development of the nervous system: the aganglionic system, as illustrated by starfish; the ganglionic system, as in flatworms; and the ganglionic chain system, as in earthworms and other segmented animals. Whereas the nervous system of echinoderms, coelenterates, etc., is undoubtedly diffuse, it does not seem quite correct to designate it as aganglionic. Aggregations of nerve cells occur in the oral regions of coelenterates and in connection with the marginal sense organs of scyphomedusae, and J. E. Smith has recently shown the presence of such aggregations in starfish where the arms meet the disk.

The final chapters treat variability in behavior as a manifestation of variation in internal physiological states and habit formation as a basis for mind. The author opposes the view, probably acceptable to most zoölogists, that mind is present in some form in even the Protozoa. He rightly disposes of certain cases of supposed associative behavior and learning in Protozoa, including the dubious experiments of the Marburg school, as examples of simple hysteresis, by which is presumably meant altered behavior as a result of altered physicochemical constitution. But perhaps all learning is the result of altered physicochemical constitution of the nervous system? The reviewer is disposed to believe that genuine learning may occur in animals below the grade of the ganglionic nervous system, but undoubtedly all alleged cases should be subjected to the most rigid critical inspection.

Although the book contains much of interest and value, it suffers from the fact that the author is unable to resolve the conflict between the mechanistic viewpoint in which he professes to believe and the organismal viewpoint that constitutes his real philosophy, as shown by various statements throughout the work.

HEREDITY AND ITS VARIABILITY,[1] BY T. D. LYSENKO TRANSLATED BY DOBZHANSKY

Reviewed by RICHARD B. GOLDSCHMIDT
University of California

SIXTEEN years ago the reviewer attended as an invited guest the All-Soviet Congress of Geneticists and Breeders. He found a unique organization of genetics applied to animal and plant breeding, with hundreds of enthusiastic workers who were working day and night on problems of fundamental and applied genetics. The driving spirits were Vavilov, in the applied fields, and Koltzoff and Serebrowsky, in fundamental genetics. Both the reviewer and his companion, Erwin Baur, were immensely impressed by what they saw. Since that time a change has occurred, of the details of which the scientific world is still incompletely informed. An agronomist, Lysenko, had succeeded in improving crops considerably by his method of yarovization, i.e., purely physiological action upon seed. This led him to the extreme idea that genetics is not able to improve domestic plants and animals, even that the basic tenets of genetics are wrong, and that the only way of changing heredity is by environmental action. It is not known how Lysenko succeeded in making his amateurish views the official viewpoint of the authorities. But it seems to be a fact that he took over the entire program of breeding, removed the geneticists, made things unpleasant also for the workers in fundamental genetics, and, generally speaking, tried to destroy what hundreds of excellent scholars had built up. This situation is difficult to understand because of our rather complete ignorance of Lysenko's own performance. The present publication is meant to supply such information to the English-speaking world, and it is welcome from the standpoint of history, although its contents would hardly have justified publication otherwise.

The pamphlet is obviously meant as a semipopular presentation of Lysenko's views—perhaps for breeders, as no scientific literature is quoted, while such amateurs as Burbank and Michurin are played up. This makes it difficult to judge whether the author is actually as completely ignorant as it seems of the science of genetics which he attacks and whether he really does not even know the classic work in his own field (graft hybrids). It is possible that he omitted scientific discussions from a book intended for the layman and thus purposely produced a discussion on a very low level.

The first part of the pamphlet presents Lysenko's views on heredity, which, to him, is "the essence of the conditions of the external environment assimilated by the plant organisms in a series of preceding generations." He develops a very simple and primitive Lamarckism shrouded in pseudophysiological language, some of it utterly muddled but some developed with the argumentation of a shrewd lawyer. As no attempt is made to bring his beliefs into line with scientific facts (except his own, reviewed below), nothing is gained by reading it but an insight into the rather naïve private beliefs of a

[1] New York: King's Crown Press, 1945. Pp. 72. $0.50.

man who either does not know or does not want to discuss the decisive facts which are incompatible with his views.

A second section informs us of his own experiments, which are supposed to prove his contentions. (Here, by the way, he allows us repeatedly a glimpse into what is probably the source of his political success: He plays up his work as a 100 per cent Soviet performance, implying that genetics is "furriners'" stuff.) The experiments seem all to be of the following type: One variety is grafted upon another, and in many cases the graft assumes characters of the scion, which are now inherited by the seed from the graft. These graft hybrids prove to Lysenko that a plastic substance enters from the scion to the graft and is incorporated into its heredity. This return to pre-experimental views on graft hybrids is presented as a dogmatic statement. We are not told that any checks have been made to exclude a chimeric growth from the subepidermal layer of the scion or that any genetical or cytological checks have been made. As we have complete information on chimeras (and one single real graft hybrid, or burdo) from the work of Baur, Winkler, Haberlandt, it would be Lysenko's duty to exclude the classic interpretation of his results (assuming their correctness) before he draws what everybody else considers wild conclusions. Maybe Lysenko did all this, but he fails to tell us; therefore his claims cannot be accepted. As presented in this pamphlet the work seems to be completely dilettantist, and the conclusions can hardly be labeled as science. For example (p. 37):

> The seeds of vegetative hybrids obtained on the scion and sown into the soil will select from the surrounding environment the conditions which are, in the last analysis, necessary for building the body of the given organism. The latter, however, is now similar to that which arose for the first time through a forced assimilation of unsuitable conditions in the graft. Thus if the scion was forced to assimilate the plastic substances from which, as a result of a series of biochemical transformations, red tomato fruits are obtained, then the seeds from these fruits, when sown, will be inclined to select from the external environment all those conditions which, after many regular transformations, will give ripe fruits of a red color.
>
> The plastic substances of the stock are an external element, food, with respect to the scion. Yet, these substances become, through assimilation, an integral part of the scion and alter its hereditary properties.

A third section presents his results with winter wheat. In principle the story is the same. A winter variety can be transformed into a hereditary spring variety by proper temperature treatment. It is difficult to find out from the data presented in the pamphlet how the experiments were performed, how the obvious sources of error were excluded, whether any genetical tests were made, whether the experiments started with pure lines tested for heterozygosity, etc. I do not think that any biologist would accept Lysenko's claims without proof that the known sources of error were excluded and that the necessary checks had been made.

The rest of the booklet contains varied data on "facts" which are supposed to disprove the basic tenets of genetics. Thus, Mendelism is "disproved" by the statement that Mendel once found a segregation of 19:20 instead of 3:1; or by the claim that a cross between a 16-chromosome plum and one with 48 chromosomes resulted in a purely paternal type with 48 chromosomes; or by claiming cases of maternal, paternal blending inheritance without even trying the elementary tests. *Sapienti sat!*

The reviewer cannot help saying that it is a saddening experience to read this pamphlet and that his sympathy goes out to the hundreds of fine biologists—both applied and fundamental—in the U.S.S.R. who have to accept silently the leadership of a man who has clearly neither the knowledge or the ability for reasoning nor the understanding of experimental procedure and check nor the self-criticism which could make him a leader, at least as far as science is concerned.

A SYMPOSIUM ON MAMMARY TUMORS IN MICE,[1] BY MEMBERS OF THE STAFF OF THE NATIONAL CANCER INSTITUTE EDITED BY FOREST RAY MOULTON

Reviewed by LEO LOEB
Washington University Medical School

THIS monograph on mammary tumors in mice is the co-operative work of members of the National Cancer Institute. All the authors have been active in cancer research. While this volume deals only with one type of cancer, namely, with carcinoma of the mammary gland of the mouse, it may very well serve as an introduction to the study of cancer in general, because the nature and development of this particular tumor have probably been analyzed by experimental methods more thoroughly than any other kind of mammalian tumor and also because the principles underlying all cancerous growths seem to be essentially the same. In a wider sense this latter statement is true also of certain tumors in birds and in some cold-blooded animals and even in plants.

To make the contents of this book more readily understandable to biologists, many of whom may not be familiar with the problems of cancer, it might be well to state briefly a few of the principal data concerning the nature and causes of this condition.

In all cases we have to deal essentially with tissues which have assumed an increased growth energy quite commonly associated with an increased mobility of the affected cells, changes which have become permanent and irreversible and are transferred to successive cell generations. These changes, furthermore, are associated with certain structural and chemical alterations of the affected tissues which tend to be in the same direction as those found in certain other noncancerous growth processes, although quantitative differences may exist in this respect between these two types of growth.

Moreover, in all cancerous growths similar factors bring about these changes, namely, growth-promoting (stimulating) factors, which, as a rule, act over long periods of time in association with genetic factors, which in many instances seem to influence the cancerous transformation largely by affecting the responsiveness of the tissues to the stimuli.

It may be assumed that, through the long-continued combined action of these two conditions, a factor is produced within the cells which propagates spontaneously, perhaps by autocatalysis, and causes the increase in growth and associated phenomena. There may, in addition, be active various secondary factors, especially nutritional ones. The effective

[1] Washington, D.C.: American Association for the Advancement of Science, 1945. Pp. 223. $3.50.

stimulating factors may be of various kinds, such as hormones, certain polycyclic hydrocarbons, radiations, mechanical factors initiating regenerative processes, some metazoic parasites, and also viruses, all acting, as a rule, in cooperation with intrinsic genetic factors.

It may be mentioned that some investigators assume that the stimulating and genetic factors, inherent in the tissue substratum, function only indirectly by making it possible for a virus to become active, which latter would then initiate the actual cancerous change; still others believe that these factors bring about preparatory conditions, which make it possible for a somatic chromosomal mutation to induce the cancerous growth.

In the "spontaneous" mammary-gland carcinoma of the mouse, the stimulating factors have been found to consist of ovarian hormones, which act in cooperation with genetically determined factors. But mammary-gland carcinoma differs from other mammalian cancers in that a third factor may be required for the cancerous transformation, namely, a virus-like substance transmitted to the young with the milk of its mother or of a foster-mother. This milk factor is effective only in co-operation with the two other factors, all acting over long periods of time. However, there has recently accumulated some evidence which seems to indicate that the presence of this milk agent is not required in all cases for the production of mammary-gland carcinoma in mice, and it may be suggested that its essential function is that of intensifying the effectiveness of the other two conditions. The milk factor cannot, therefore, be considered as the real "cancer-inciter" and the stimulating and genetic factors as possessing merely a preparatory function.

All these various aspects of cancer research are treated in this book, mainly with respect to the mammary-gland carcinoma of the mouse. There is, first, a general and historical Introduction by Shimkin. This is followed by four articles dealing with the structural characteristics of the tissue substratum, the mammary gland of the mouse, during its transition from the normal to the cancerous state. These articles, written by Dalton, Dunn, Algire, and Chalkley, are, on the whole, complete and instructive. All are based on the study of microscopic material by the authors and not only on a study of the literature. However, it seems to the reviewer that, in the chapter on the morphology and histogenesis of mammary tumors, the most frequent and noteworthy precancerous change, namely, the coiling of the end ducts, might have been mentioned. The next chapter by Heston is a full and clear presentation of the genetics of mammary-tumor mice; it is chiefly the more recent investigations in this field which are discussed, without much consideration being given to the earlier research, although the main conclusions arrived at in this earlier work are still perfectly valid at the present time. This chapter will be of special interest to the geneticists. The next four chapters are of particular value to the student of the causes and nature of cancer, since they deal with the relation of hormones to the mammary cancer in mice (Shimkin), with the milk agent (Andervont), with the significance of diet and some other environmental factors (Morris), and with the chemistry of mammary tumors (Greenstein). These authors—as well as some of the others—have made noteworthy contributions to cancer research. The next chapter by Dyer discusses the experimental treatment of mammary tumors—a difficult subject which has, on the whole, been

adequately presented; but reference to some earlier findings concerning the development of a tissue immunity on the part of the cancer cells against the therapeutic substances used might have been made. Shimkin closes the book with a good, although perhaps somewhat overcautious, review of some of the results obtained in cancer research, together with a discussion of the possible implications for man.

It is to be expected that, in a field in which research is so active, different investigators should differ in some of their interpretations and evaluations of the established data. Taken as a whole, it is an excellent work, and it can be highly recommended to the biologist who wishes to become acquainted with so remarkable a biological phenomenon as cancerous growth. To him this volume may serve as a valuable introduction.

VIRUS AS ORGANISM,[1] BY FRANK MacFARLANE BURNET

Reviewed by WILLIAM BALAMUTH
Northwestern University

IN THE short space of fifty years a tremendous mass of data has accumulated bearing upon the biological action and physicochemical properties of filterable disease agents, generally termed "viruses." Several recent reviews have emphasized chemical aspects of virology, but a corresponding biological synthesis has been noticeably lacking. This gap has been lessened by Burnet's excellent, concise treatment of *Virus as Organism*. The book represents an expansion of the Dunham Lectures at Harvard University. It has the special merit of joining a thoughtful interpretation of viruses as living organisms to well-documented references to recent developments concerning some human viral diseases with which the author's own research has been concerned. It is strongly recommended reading for all biologists who have felt the need of an authoritative, stimulating orientation of viruses in the scale of living and nonliving matter.

The title indicates Burnet's basic theme, that "viruses are micro-organisms which have evolved by parasitic degeneration from larger micro-organisms, many of them in all probability from bacteria." The author justifies his consistently biological interpretation as a necessary condition in reconciling the present divergence between clinical and experimental work and general biological thought.

The initial section of the book (chaps. i–iii) analyzes certain basic viral properties: reproduction, variation, survival, evolution, and host-parasite interactions. Viruses apparently reproduce by the replication of subcellular entities, basically representing a special example of protein synthesis. In this connection Burnet points out the unreal basis for the debate as to whether smaller viruses are to be regarded as protein molecules or living organisms, since protein synthesis is an exclusively biological capacity about which little is known thus far.

Heritable variations unquestionably occur among viruses, as well as among higher organisms. The author visualizes these as instances of genetic mutation,

[1] Cambridge, Mass.: Harvard University Press, 1945. Pp. 134. $2.00.

with selective survival and overgrowth by one or more mutants providing the basis for mass transformation of strains. The staggering number of individuals and the short generation-time involved in a dynamic host-parasite environment provide an excellent test example of Darwinian natural selection.

The evolutionary transition to viruses probably involved a sequence from free-living microscopic saprophyte to facultative parasite to obligatory parasite with subsequent degenerative adaptation, until there remained "nothing but a shadowy, self-replicating residuum of genetic mechanism." Beadle's work on Neurospora is cited as an example of how gene mutations can cause progressive deficiency in biochemical syntheses, while the intracellular environment of viruses would offer no obstacle to continued survival of such degenerate entities, presumably even at the level of highly complex protein molecules. Burnet supports the view that human viral diseases probably have evolved during the historical period since man adopted the gregarious habit and thus made possible large aggregations of susceptible host-individuals. Yellow fever is an older disease fitting this picture, while examples of more recent diseases possibly still undergoing adaptation are psittacosis and certain forms of encephalitis.

Host-parasite interactions in viral diseases are not well understood as yet. Burnet notes that viruses grow only inside of living cells and, in general, are restricted to specific types of cells. The most important immune reaction unquestionably involves production of specific antibody. However, prolonged immunity is characteristic only of those diseases (like yellow fever) in which virus is present in high concentration in the blood (10^{10} infecting doses per cubic centimeter in yellow fever) and in which reinfecting particles must be exposed to circulating antibody before reaching susceptible cells.

The remainder of the book (chaps. iv–ix) treats six human viral diseases from a consistently ecological and evolutionary point of view; these diseases include herpes simplex, poliomyelitis, psittacosis, smallpox and similar diseases, yellow fever, and influenza. In each instance the virus is considered with respect to its mode of survival, characteristic range of variation, and possible evolution; and for the human the role of age and immune responses is stressed. The current status of research on each of these diseases is set forth clearly and in an interesting, narrative manner.

In a concluding section Burnet points out that the future of human viral diseases is unpredictable, relying as it does upon random mutations of viral particles, the impossibility at present of controlling respiratory infections, and the existence of vast numbers of potential natural reservoirs of infection in surrounding arthropods, birds, and mammals.